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(54) Title: HUMAN ADENOSINE RECEPTOR ANTAGONISTS

(57) Abstract

The invention concerns the use of compounds, identified through the use of recombinant human adenosine receptors A1, A2a, A2b and A3, and functional assays, to specifically modulate the physiologic role of adenosine activation of its various receptors.

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TITLE OF THE INVENTION
HUMAN ADENOSINE RECEPTOR ANTAGONISTS

RELATED APPLICATIONS

This application is a continuation-in-part of USSN 08/145,437, filed on 10/29/93, pending.

GOVERNMENT SUPPORT

This invention was made with government support under RO1HL37942 grant awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION:

The present invention concerns the use of compounds identified as specific modulators of adenosine's physiological actions. The pharmacology of these compounds is characterized through the use of cloned human adenosine receptors of the A1, A2a, A2b and A3 class and their subtypes. Compounds identified as antagonists of the A3 adenosine receptor subtype are useful in preventing mast cell degranulation and are therefore useful in the treatment or prevention of disease states induced by activation of the A3 receptor and mast cell activation. These disease states include but are not limited to asthma, myocardial reperfusion injury, allergic reactions including but not limited to rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, other autoimmune diseases and inflammatory bowel diseases.

2. BACKGROUND:

Adenosine is a naturally occurring nucleoside which exhibits diverse and potent physiological actions in the cardiovascular, nervous, pulmonary, renal and immune systems. Adenosine has been demonstrated to terminate superventricular tachycardia through blockage of atrioventricular nodal conduction (J.P. DiMarco, et al., (1985) J. Am. Col. Cardiol. 6:417-425, A. Munoz, et al., (1984) Eur. Heart J. 5:735-738). Adenosine is a potent vasodilator except in the kidney and placenta (R.A. Olsson, (1981) Ann. Rev. Physiol. 43:385-

395). Adenosine produces bronchoconstriction in asthmatics but not in nonasthmatics (Cushly et al., 1984, Am. Rev. Respir. Dis. 129:380-384). Adenosine has been implicated as a preventative agent and in treatment of ventricular dysfunction following episodes of regional or global ischemia (M.B. Forman and C.E. Velasco (1991) Cardiovasc. Drugs and Therapy 5:901-908) and in cerebral ischemia(M.C. Evans, et al., (1987) Neurosci. Lett. 83:287, D.K.J.E.,Von Lubitz, et al., (1988) Stroke 19:1133).

Dog A1 and A2a adenosine receptors were the first adenosine receptors to be cloned. See F. Libert, et al., (1989) Science 244:569-572, C. Maennant, et al., Biochem. Biophys. Res. Comm., (1990) 173:1169-1178, and F. Libert, et al. (1991) EMBO J. 10:1677-1682. The rat A1 adenosine receptor was cloned by L.C. Mahan, et al., (1991) Mol. Pharm. 40:1-7 and S.M. Reppert, et al., (1991) Mol. Endocrin. 5:1037-1048, the rat A2a by Fink et. al., (1992) Mol. Brain Res. 14:186-195, and the rat A2b by Stehle et al. (1992) Mol. Endocrinol. 6:384-393. Cloning of the rat A3 adenosine receptor was reported by Meyerhof et al., (1991) FEBS Lett. 284:155-160 and Zhou et al., (1992) PNAS USA 89:7432-7436. Cloning of the sheep A3 adenosine receptor has been reported by Linden et al., (1993) Mol. Pharm. 44:524-532. Cloning of the human A1, A2a, A2b and A3 receptors were reported in GB 2264948-A (9/15/93). The human A1 adenosine receptor differs by 18 amino acids from the dog A1 sequence and 16 amino acids from the rat A1 sequence. The human A2a adenosine receptor differs by 28 and 71 amino acids, respectively from the dog and rat A2a sequences. The amino acid sequence for the human A3 receptor is 72% identical with the rat A3 receptor and 85% identical with the sheep A3 receptor sequences.

The actions of adenosine are mediated through G-protein coupled receptors, the A1, A2a, A2b and A3 adenosine receptors. The adenosine receptors were initially classified into A1 and A2 subtypes on the basis of pharmacological criteria and coupling to adenylate cyclase (Van Caulker, D., Muller, M. and Hamprecht, B. (1979) J. Neurochem. **33**, 999-1003.). Further pharmacological classification of adenosine

receptors prompted subdivision of the A2 class into A2a and A2b subtypes on the basis of high and low affinity, respectively, for adenosine and the agonists NECA and CGS-21680 (Bruns, R.F., Lu, G.H. and Pugsley, T.A. (1986) Mol. Pharmacol. **29**, 331-346; Wan, W., Sutherland, G.R. and Geiger, J.D. (1990) J. Neurochem. **55**, 1763-1771). The existence of A1, A2a and A2b subtypes has been confirmed by cloning and functional characterization of expressed bovine, canine, rat and human receptors. A fourth subtype, A3, had remained pharmacologically undetected until its recent identification by molecular cloning. The rat A3 sequence, tgpcr1, was first cloned from rat testis by Meyerhoff et al. (see above). Subsequently, a cDNA encoding the identical receptor was cloned from striatum and functionally expressed by Zhou et al. (see above). When compared to the other members of the G-protein coupled receptor family, the rat sequence had the highest homology with the adenosine receptors (> 40% overall identity, 58% within the transmembrane regions). When stably expressed in CHO cells, the receptor was found to bind the radioligand ^{125}I-APNEA (N^6-2-(4-amino-3-iodophenyl)ethyladenosine) and when transfected cells were treated with adenosine agonists, cyclic AMP accumulation was inhibited with a potency order of NECA = R-PIA > CGS21680. The rat A3 receptor exhibited a unique pharmacology relative to the A1 and A2 adenosine receptor subtypes and was reported not to bind the xanthine antagonists 1,3-dipropyl-8-phenylxanthine (DPCPX) and xanthine amine congener (XAC). Messenger RNA for the rat A3 adenosine receptor is primarily expressed in the testis.

The sheep homolog of the A3 receptor was cloned from hypophysial pars tuberalis (see Linden et al. above). The sheep receptor is 72% identical to the rat receptor, binds the radioligand ^{125}I-ABA and is also coupled to inhibition of cyclic AMP. The agonist affinity order of the sheep receptor is I-ABA > APNEA> NECA $\geq$ R-PIA >> CPA. The pharmacology of xanthine antagonists was extensively studied and the sheep receptor was found to exhibit high affinity for 8-phenylxanthines with para-acidic substitutions. In contrast to the rat transcript, the expression of the sheep A3 adenosine receptor transcript

is widespread throughout the brain and is most abundant in the lung and spleen. Moderate amounts of transcript are also observed in pineal and testis. Thus, because the published literature provides an inconsistent profile of adenosine A3 receptor pharmacology and tissue distribution, it was not possible to predict the pharmacology or tissue distribution of the human A3 adenosine receptor.

Although the human A1, A2a and A2b adenosine receptor cDNAs have been cloned, the tissue distribution of human adenosine receptor transcripts has not been previously presented. This patent disclosure describes the characterization of a human A3 adenosine receptor subtype, the pharmacological profile of the human A3 adenosine receptor, and the tissue distribution of human A1, A2a, A2b and A3 adenosine receptor transcripts.

Based on the use of these cloned receptors, an assay has been described to identify adenosine receptor agonists and antagonists and determine their binding affinity (see GB 2 264 948 A, published 9/15/93; see also R.F. Bruns, et al., (1983) Proc. Natl. Acad. Sci. USA 80:2077-2080; R.F. Bruns, et al.,(1986) Mol. Pharmacol. 29:331-346; M.F. Jarvis, et al. (1989) J. Pharma. Exp. Therap. 251:888-893; K.A. Jacobson et al., (1989) J. Med. Chem. 32:1043-1051).

Adenosine receptor agonists, antagonists and binding enhancers have been identified and implicated for usage in the treatment of physiological complications resulting from cardiovascular, pulmonary, renal and neurological disorders. Adenosine receptor agonists have been identified for use as vasodilators ((1989) FASEB. J. 3(4) Abs 4770 and 4773, (19910 J. Med. Chem. (1988) 34:2570), antihypertensive agents (D.G. Taylor et al., FASEB J. (1988) 2:1799), and anti-psychotic agents (T.G. Heffner et al., (1989) Psychopharmacology 98:31-38). Adenosine receptor agonists have been identified for use in improving renal function (R.D. Murray and P.C. Churchill,(1985) J. Pharmacol. Exp. Therap. 232:189-193) . Adenosine receptor allosteric or binding enhancers have shown utility in the treatment of ischemia, seizures or hypoxia of the brain (R.F. Bruns, et al. (1990) Mol. Pharmacol. 38:939-949; C.A. Janusz, et al., (1991)

Brain Research 567:181-187). The cardioprotective agent, 5-amino-4-imidazole carboxamide (AICA) ribose has utility in the treatment of ischemic heart conditions, including unstable angina and acute myocardial infarction (H.E. Gruber, et al. (1989) Circulation 80: 1400-1414).

8-phenylxanthines, methods of their synthesis and their use in human and veterinary therapy for conditions associated with the cell surface effects of adenosine have been described (EP 0 203 721, published 12/3/86). However, this publication is silent as to adenosine receptor subtypes and subtype specificity of disclosed compounds. In WO 90/00056, a group of 1,3-unsymmetrical straight chain alkyl-substituted 8-phenylxanthines were described as being potent bronchodilators. This disclosure is likewise silent as to the subtype specificity of disclosed compounds.

Methods of treating conditions related to the physiological action of adenosine have, to date, proven inferior due to the presence of multiple subtypes present in the animal tissue utilized (R.F. Bruns et al., (1986) Mol. Pharm. 29:331-346) and the differences between species in the affinity for adenosine analogs and the physiological effects of adenosine (Ukera, et al., (1986) FEBS Lett, 209:122-128).

Through the use of homogeonous, recombinant adenosine receptors, the identification and evaluation of compounds which have selectivity for a single receptor subtype has now been accomplished. Moreover, because of the variable effects of adenosine documented in other species, the utilization of human adenosine receptor subtypes is advantageous for the development of human therapeutic adenosine receptor agonists, antagonists or enhancers. The instant patent disclosure defines compounds which unexpectedly exhibit selective binding affinity for the human A3 adenosine receptor and therefore provides a method of using such compounds which overcomes the disadvantages of using compounds of uncharacterized specificity, by specifically blocking the activities mediated through the activation of the A3 receptor subtype without substantially blocking the activities of the other adenosine receptor subtypes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1	Full length amino acid sequence of human A1 adenosine receptor.
Figure 2A-B	Full length nucleotide sequence of the cloned human A1 adenosine receptor complementary DNA depicted from the 5' to 3' terminus.
Figure 3	Full length amino acid sequence of human A2a adenosine receptor.
Figure 4A-B	Full length nucleotide sequence of cloned human A2a adenosine receptor complementary DNA depicted from the 5' to 3' terminus.
Figure 5	Full length amino acid sequence of human A2b receptor.
Figure 6A-B	Full length nucleotide sequence of cloned human A2b adenosine receptor complementary DNA depicted from the 5' to 3' terminus.
Figure 7	Saturation binding of [^{3}H]-cyclohexyladenosine (CHA) to human A1 adenosine receptor in COS7 assay.
Figure 8	Saturation binding of [^{3}H]-CGS21680 to human A2a adenosine receptor in COS7 assay.
Figure 9	Full length amino acid sequence of human A3 adenosine receptor.
Figure 10A-B	Full length nucleotide sequence of the cloned human A3 adenosine receptor complementary DNA depicted from the 5' to 3' terminus.

Figure 11 (A) Equilibrium binding of ^{125}I-ABA to membranes prepared from A3 stable transfected CHO cells shows specific (•) and nonspecific (o) binding. Nonspecific binding was measured in the presence of 1 μM I-ABA.
(B) Scatchard transformation of the specific binding.

Figure 12A-B Competition by agonists and antagonists for ^{125}I-ABA binding to membranes prepared from stably transfected CHO cells expressing the human A3 adenosine receptor. Agonists (top panel), (•) NECA, (o) R-PIA, (•) CPA, ( ) S-PIA; antagonists (bottom panel), (•) I-ABOPX, (•) BW-A1433, (o) XAC, ( ) DPCPX.

Figure 13A-F Competition by antagonists of NECA-inhibited cyclic AMP accumulation in CHO cells stably expressing the human A3 adenosine receptor. Dose response curves to NECA measured in the absence or presence of two concentrations of BW-A1433, XAC and I-ABOPX. The ED50's were used to construct Schild plots. Each figure is representative of 2-3 experiments.

Figure 14A-B Northern blot analysis of the four human adenosine receptor subtypes. (A) 5 μg poly(A)$^+$ RNA from various human tissues probed with HS-21a. The two blots shown were transferred, hybridized and exposed separately. (B) 7.5 μg poly(A)$^+$ RNA from various human tissues probed with either A1, A2a, or A2b. Each blot was transferred and exposed separately.

Figure 15(A)-(E) Structures of xanthine derivatives.

Figure 16 An image showing the abluminal surface of an arteriole

after staining with methylene blue, clearly labeling adherent mast cells, some of which are degranulated from prior exposure to adenosine.

Figure 17 Change in microvessel diameter with A3 agonist.

Figure 18 Peak concentration-response curves for untreated vessels exposed to compound 48/80 (n = 4). Values are mean ± s.e. for the peak luminal diameter attained within one minute of compound 48/80 application.

Figure 19 Peak concentration-response curves for control arterioles and arterioles pretreated with cromolyn. "Initial" indicates baseline tone before pretreatement. *, $p < 0.05$.

Figure 20 Abolition of agonist induced contractile response by pre-treatment with an adenosine A3 receptor specific antagonist.

SUMMARY OF THE INVENTION

The invention concerns the use of compounds, identified through the use of recombinant human adenosine receptors A1, A2a, A2b and A3, and functional assays, to specifically modulate the physiologic role of adenosine activation of its various receptors.

The human A3 adenosine receptor was cloned from a striatal cDNA library using a probe derived from the homologous rat sequence. The cDNA encodes for a protein of 318 amino acids and exhibits 72% and 85% overall identity with the rat and sheep A3 adenosine receptor sequences, respectively. Specific and saturable binding of the adenosine receptor agonist, ^{125}I-N^6-aminobenzyladenosine (^{125}I-ABA) was measured on the human A3 receptor stably expressed in CHO cells with a K_D = 10 nM. The potency order for adenosine receptor agonists was determined to be N-ethylcarboxamidoadenosine (NECA) ≥ R-phenylisopropyladenosine (R-

PIA) > N^6-cyclopentyladenosine (CPA) > S-phenylisopropyladenosine (S-PIA). The human receptor was found to be blocked by xanthine antagonists. A partial listing of the pharmacology is that the potency order of antagonists is I-ABOPX > 1,3-dipropyl-8-(4-acrylate) phenylxanthine (BW-A1433) ≥ xanthine amino cogener (XAC) >> 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). Adenosine, NECA, R- and S-PIA and CPA inhibited forskolin-stimulated cAMP accumulation by 30-40% in the stably transfected CHO cells; I-ABA is a partial agonist. When measured in the presence of antagonists, the dose response curves of NECA-induced inhibition of forskolin-stimulated cAMP accumulation were right-shifted. Antagonist potencies determined by Schild analyses correlated well with those established by competition for radioligand binding. The tissue distribution of transcripts for all of the human adenosine receptor subtypes was compared. The A3 adenosine receptor transcript is widespread, and in contrast to the A1, A2a and A2b transcripts, the most abundant expression is found in the lung and liver. By comparison, the rat A3 adenosine receptor transcript is primarily expressed in testis and the sheep transcript is most abundant in the lung, spleen and pineal. The human tissue distribution of A3 mRNA is more similar to the widespread profile found in sheep than to the restricted profile found in the rat. Numerous physiological effects of adenosine may be mediated by A3 adenosine receptors in man.

ABBREVIATIONS

[^{3}H]-CHA, [^{3}H]-cyclohexyladenosine; [^{3}H]-NECA, [^{3}H]-5'-N-ethyl-carboxamido-adenosine; ^{125}I-ABA, N^6-(4-amino-3-^{125}iodobenzyl)adenosine; ^{125}I-APNEA, N^6-2-(4-amino-3-^{125}iodophenyl)ethyladenosine; NECA, N-ethylcarboxamidoadenosine; CGS21680, 2-[4-(2-carboxyethyl)phenyl]ethylamino-5'-N-ethylcarboxamidoadenosine; (R,S)-PIA, (R,S)-N^6-phenyl-2-propyladenosine; CPA, N^6-cyclopentyladenosine; I-ABOPX, (3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenyl-1-prpopylxanthine; BW-A1433, 1,3-dipropyl-8-(4-acrylate)phenylxanthine; XAC, xanthine

amine cogener; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GTPγS, guanosine 5'-O-3-thiotriphosphate; Gpp(NH)p, 5'-guanylimidodiphosphate; G protein, guanine nucleotide-binding proteins.

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DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method for achieving specific blockade of the A3 subtype of the adenosine receptor. We have discovered that adenosine, adenosine metabolites and other A3 adenosine receptor agonists induce mast cell degranulation in an animal model and that this can be prevented by selective antagonists of the A3 receptor. The release of enzymes, bioactive amines and arachidonic acid metabolites following mast cell activation causes vasoconstriction, edema, leukocyte accumulation, and ultimately, tissue damage. Mast cell degranulation is a component of: myocardial reperfusion injuryu, hypersensitivity reactions (asthma, allergic rhinitis, and urticaria), ischemic bowel disease, autoimmune inflammationa, and atopic dermatitis. The invention consists of the use of any of a series of highly specific A3 adenosine receptor antagonists to treat or prevent these diseases and pathologic effects that result from mast cell degranulation.

Other physiologic effects induced through activation of the A3 adenosine receptor are also amenable to modulation through blockade of A3 adenosine mediated responses in basophiles, eosinophiles and other immune cells. Mast cell degranulation is clearly involved in the pathophysiology of allergies such as asthma. Autoimmune diseases are also characterized by immune reactions which attack targets, including self-proteins in the body such as collagen, mistaking them for invading antigens. The resulting damage, caused at least in part by mast cell degranulation, is amenable to treatment by the method of this invention which comprises administration of selective A3 adenosine

receptor antagonists effective to inhibit mast cell degranulation. Among these types of diseases, all of the following, but not limited to these, are amenable to treatment by the method of this invention: Addison's disease (adrenal), autoimmune hemolytic anemia (red cells), Crohn's disease (gut), Goodpasture's syndrome (kidney and lungs), Grave's disease (thyroid), Hashimoto's thyroiditis (thyroid), idiopathic thrombocytopinic purpura (platelets), Insulin-dependent diabetes militus (pancreatic beta cells), multiple sclerosis (brain and spinal cord), myasthenia gravis (nerve/muscle synapses), Pemphigus vulgaris (skin), pernicious anemia (gastric parietal cells), poststreptococcal glomerulonephritis (kidney), psoriasis (skin), rheumatoid arthritis (connective tissue), scleroderma (heart, lung, gut, kidney), Sjogren's syndrome (liver, kidney, brain, thyroid, salivary gland), spontaneous infertility (sperm), systemic lupus erythematosus (DNA, platelets, other tissues).

The method of this invention provides a means for preventing or treating disease states associated with vascular constriction induced through activation of the A3 subtype of the adenosine receptor. The method comprises contacting said receptor in the vasculature with an amount of a compound which selectively blockades activation of the A3 adenosine receptor subytpe. In one embodiment of the invention, this blockade occurs on granulocytes, including mast cells, exhibiting the A3 adenosine receptor. In one embodiment of the invention, xanthine or a xanthine derivative is used to effect a reduction in vasoconstriction in the vasculature without any substantial effect (binding or blockade) of the A1, A2a or A2b subtypes of the adenosine receptor. The method extends to the treatment or prevention of disease states mediated through activation of the A3 subtype of the adenosine receptor on mast cells. Prevention of mast cell degranulation through blockade of the A3 subtype of the adenosine receptor by contacting mast cells with an inhibitory effective amount of a xanthine or xanthine derivative specific for the A3 receptor subtype therefore also forms part of this invention. Disease states associated with A3 adenosine receptor activation and mast cell degranulation include, but are not

limited to asthma, myocardial reperfusion injury, allergic reactions including but not limited to rhinitis, asthma, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases.

Compounds having specific affinity for the A3 adenosine receptor subtype are identified through the pharmacology displayed in binding to isolated receptors of the various subtypes. Many compounds have been identified broadly as antagonists of the adenosine receptor. However, the subtype specificity of these compounds, in particular the pharmacology of the primate A3 receptor subtype, was not known prior to the instant patent disclosure. This disclosure defines xanthines and xanthine derivatives displaying potent and specific A3 subtype specificity. This disclosure also defines the unexpectedly similar tissue distribution and pharmacology of the human and sheep A3 receptors. Because of this discovered similarity, it is now possible to utilize known sheep A3 pharmacology to predict primate pharmacology. In addition, this disclosure demonstrates the functional effects of using A3 specific compounds in blocking adenosine induced effects. Microvasculatrue contraction is inhibited, intracellular cAMP reduction, which is normally induced by A3 receptor agonism, is blocked and mast cell degranulation is inhibited.

A cDNA from human striatum designated HS-21a that encodes a human A3 adenosine receptor has been cloned. The cDNA is homologous with rat (5,6) and sheep clones (7), and all three sequences encode receptors that couple adenosine induced inhibition of cAMP accumulation when stably expressed in CHO cells. ^{125}I-ABA, previously used as a radioligand for A1 adenosine receptors (12), was found to be suitable for detecting recombinant CHO cells expressing human A3 adenosine receptors.

The sheep A3 adenosine receptor transcript is widely distributed, with high levels found in lung and spleen and moderate levels found in brain, pineal and testis. In marked contrast, the rat A3 adenosine receptor transcript is found primarily in testis. Therefore, it was of great interest to determine the tissue distribution of the human

A3 adenosine receptor transcript. We have discovered that the human transcript is found to be more similar to the sheep than the rat homolog, with high expression in lung, moderate expression in brain and low expression in testis.

The rat A3 adenosine receptor differs from the human and sheep receptors in that it was reported not to bind the xanthine antagonists, XAC and DPCPX (6). The sheep and now the human A3 adenosine receptors have been found to bind both antagonists and also have high affinity for 8-substituted xanthines having acidic substitutions. At the same time that the acidic substitutions increase the binding affinity of these compounds for the A3 receptor, they decrease the affinity for the A1, A2a and A2b subtypes. A limited number of xanthine analogs were evaluated in the pharmacological characterization of the rat A3 receptor and it was reported that these compounds do not significantly bind to the rat A3 receptor. The opposite is true in the case with the sheep A3 homolog. We have now discovered that the human A3 receptor has a high affinity for this class of compounds.

A few significant differences in ligand binding and transcript expression exist between the human and sheep receptors. I-ABA appears to be a full and partial agonist, respectively, for lowering cyclic AMP in CHO cells transfected with sheep and human receptors. The human receptor has higher affinity for CPA in comparison to the sheep receptor. An agonist affinity order of I-ABA > NECA > R-PIA > S-PIA >> CPA was established for the sheep receptor (7). By comparison, the human receptor has a generally higher affinity for all of the agonists and a preference for CPA over S-PIA, resulting in an agonist profile of I-ABA > NECA > R-PIA > CPA > S-PIA.

The antagonist affinity order profiles are similar between human and sheep receptors, however, the human homolog exhibits a higher affinity for XAC. Since 8-phenylxanthines with para-acidic residues were found to bind with high affinity to sheep A3 adenosine receptors (7), we evaluated acidic 8-phenylxanthines as human A3 adenosine receptor antagonists. Included among these are compounds with various 3-substitutions that were evaluated previously as potent

antagonists of A1 adenosine receptors (13). One of these compounds, I-ABOPX, was found to have the highest affinity as an A3 adenosine receptor antagonist, with a K_i of 18 nM for the human receptor and 2 nM for the sheep receptor.

The potency order profiles of agonist and antagonist binding to the A3 receptor differ substantially from the profiles established for the other cloned human adenosine receptor subtypes (4). Pharmacologically, the A3 receptors appear to more closely resemble A1 than the A2 adenosine receptors. This is consistent with the fact that the human A3 sequence is more similar to the A1 subtype (identity score 49%) than to the A2a and A2b subtypes. All subtypes of human adenosine receptors are blocked by xanthine antagonists such as BW-A1433, XAC and DPCPX, but differ in their affinities and potency order profiles for these ligands. The A1 subtype has high affinity for agonists with saturated rings in the N^6 position of the adenine ring, and xanthine antagonists with saturated rings in the C^8 position.

The human A3 receptor has a slightly higher affinity for ligands with unsaturated than saturated rings in the N^6 position of agonists (R-PIA > CPA) and in the C^8 position of xanthines (BW-A1433 and XAC > DPCPX). On the basis of structure-activity relationships of A1 agonists and antagonists, a model has been proposed in which the N^6-substituents of the adenine ring can be superimposed upon the C^8-regions of xanthines (27,28). A similar relationship has been suggested to exist for the sheep A3 receptor since parallel changes in potency for agonists and antagonists were observed when the N^6 and C^8 positions, respectively were substituted with unsaturated or saturated rings (7). The human A3 receptor was found to also exhibit corresponding changes in agonist and antagonist affinities when saturated substitutions were introduced at the N^6 and C^8 positions.

In a preferred embodiment of this invention, an A3 adenosine receptor antagonist will have a pKi for the A3 sutype of 7 or greater, and a pKi for other adenosine receptor subtype of 6 or less. From this disclosure, it will be apparent to those skilled in the art that xanthine compounds having the following characteristics are preferred:

An acidic, aromatic substitution at the 8 position of the xanthine (the acidity decreases affinity to A1 subtype to below a pKi of about 6, and the aromatic substitution reduces the affinity for the A2 subtype to below about 6). By further modifying the xanthine to include an aromatic, and preferably a halogenated aromatic at the xanthine 3 position, these trends in binding affinity are accentuated.

A listing of the antagonist profiles for a number of these compounds is shown in Table 1, and the structures of these compounds is shown in the figures:

TABLE 1

	Human A3	HUMAN A1
Drug	Pki	Pki
AGONISTS		
1. IABA	8.0	8.80
2. NECA	7.59	•
3. R-PIA	7.47	8.53
4. APNEA	7.22	8.05
5. CPA	6.80	9.15
6. S-ENBA	4.86	•
ANTAGONISTS		
1. IABOPX	8.13	6.12
2. XAC	7.31	•
3. BW-A1433	7.00	•
4. SPX	5.99	•
5. CPX	5.36	9.15
6. SPT	4.95	5.60
7. THEO.	4.23	•

This human data can be compared with sheep data as follows in table 2:

TABLE 2

Sheep S-17 "A3" (CHO cells)
Kd (^{125}I ABA) = **5.5** nM
Bmax = **527** fmol/mg protein

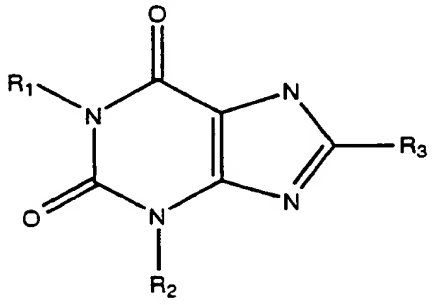
Drug	**pKi**
1. BW-A934	9.04
2. IABOPX	8.23
3. BW-A857	7.71
4. BW-1433	7.69
5. ABOPX	7.65
6. BW-B40	7.48
7. BW-215	7.26
8. XAC	6.77
9. CPX or DPCPX	4.30

Table 3. Comparison of agonist and antagonist affinities for the human A3 adenosine receptor determined in binding and functional cAMP assays.

	BINDING pK_D or pK_i	cAMP pK_A or pK_i
Agonists		
IABA	8.0	7.46
NECA	7.59	6.89
R-PIA	7.47	6.14
CPA	7.05	6.8
S-PIA	6.49	6.04
Antagonists		
IABOPX	7.74	8.13
XAC	7.15	7.66
BW-A1433	7.26	7.0
DPCPX	6.12	5.36

The log ED50 (pK_A) of agonists to inhibit cAMP accumulation and the K_i of antagonists and I-ABA was determined by Schild analysis.

From these studies, the use of a class of xanthines and their derivatives having the following characteristics is defined as having selective binding properties for the primate (including human) A3 adenosine receptor:

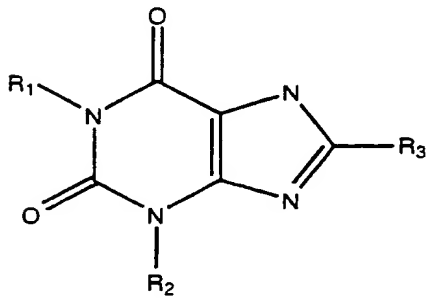


wherein R_1, R_2, and R_3, independently, are as defined below:

R_1	R_2	R_3
alkyl	aryl	acidic aryl
alkenyl	substituted aryl	substituted acidic aryl
cycloalkyl	hetero-aryl	
	substituted hetro-aryl	

wherein:
alkyl, alkenyl, cycloalkyl is substituted or unsubstituted
aryl is benzyl, phenyl;
substituted aryl is an aryl substituted with an alkyl, amino or halogen; and
acidic aryl is an aryl substituted with a carboxylate, oxyacetate, acrylate, sulphonate, phosphonate, or tetrazol.

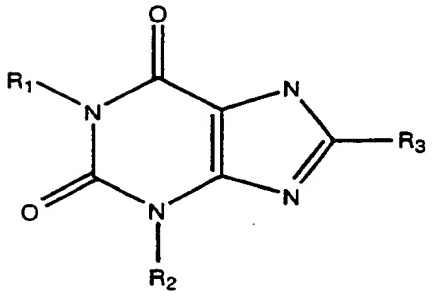
In a preferred embodiment of this invention, the xanthine is:



wherein R1, R2, and R3, independently, are as defined below:

R1	R2	R3
lower alkyl	benzyl halogenated benzyl amino-benzyl halogenated amino-benzyl.	benzyl-acid

In a futher embodiment, the xanthine is:



wherein R1, R2, and R3, independently, are as defined below:

R1	R2	R3
-C_3H_7	-C_3H_7	-CH_2-C_6H_4-O-acid
-CH_3	-benzyl	-CH_2-COO^-
-C_2H_5	-halogenated benzyl -aminobenzyl -halogenated aminobenzyl	-indole

wherein said acid is -indole, -carboxylate, sulphonate, phosphonate. In specific embodiments of the invention, the xanthine is selected from the group consisting of IABOPX, BW-A1433, BW-934, BW-A215.

A comparison of the distribution among tissues of human adenosine receptor transcripts suggests that the A1, A2a, A2b and A3

subtypes are all expressed in a number of tissues, but the pattern of transcript distribution is variable. Within the four tissues analyzed, A1 and A2a adenosine receptor transcripts are highly expressed in the brain. In contrast, the human A3 adenosine receptor transcript is most abundant in the lung and liver.

Prior to the instant patent disclosure, the physiological role of the A3 adenosine receptor was not defined. Recently, a receptor exhibiting an agonist pharmacological profile thought to be characteristic of the rat A3 subtype and insensitive to blockade by 8-(para-sulfophenyl)theophylline, was suggested to mediate in vivo hypotension in the angiotensin II-supported circulation of the pithed rat (30). The effect of 8-(para-sulfophenyl)theophylline on the cloned rat A3 receptor has not been evaluated and further pharmacological characterization is required to determine if the rat receptor binds this antagonist. 8-Para-sulfophenyltheophylline has broad action on A1, A2a and A2b adenosine receptor subtypes (2), and also blocks both the human and sheep A3 receptors. Another possible physiological role for the A3 adenosine receptor subtype in reproduction and spermatogenesis has been proposed on the basis of the abundant transcript found in rat testis and the in situ localization of mRNA within the central luminal regions of seminiferous tubules where sperm maturation occurs (5). It is possible that A3 adenosine receptors also are involved in the maturation of sperm in human and ovine testis, since low to moderate levels of receptor transcript are found in these species, respectively.

The abundant mRNA observed in the human and sheep lung is evidence that the A3 adenosine receptor subtype mediates a physiological action in the pulmonary system. Adenosine has been shown to mediate both vasodilation and vasoconstriction (31,32) in the pulmonary vasculature. In asthmatics, but not in normal patients, adenosine produces bronchoconstriction which can be antagonized by theophylline (33). The establishment of the pharmacological profile for the A3 receptor in both the human and the sheep, and the availability of subtype selective ligands facilitates the identification of the physiological functions mediated by the A3 adenosine receptor subtype and the

treatment of disease states mediated through agonism of this receptor subtype.

Adenosine has been shown to produce bronchoconstriction in asthmatics but not in nonasthmatics, demonstrating that adenosine plays a role in the etiology of this disease state (Cushly et al., 1984, AM. Rev. Respir. Dis. 129:380-384). Adenosine mediated bronchoconstriction in asthmatics is blocked by a combination of histamine and leukotriene antagonists (Bjorck et al., Am. Rev. Resp. Dis. 1992, 145:1087-1091). This indicates that adenosine acts by releasing histamine, leukotriene and other agents from mast cells or other cells that contain these allergic mediators. It is therefore within the ambit of this invention that compounds identified herein as being useful to selectively antagonize the A3 adenosine receptor are used in conjunction with other therapies. This includes co-administration of anti-histamine, leukotriene blockade or other anti-allergic mediator therapies and A3 specific antagonists. The presence of the rat A3 adenosine receptor on rat RBL-2H3 mast cells, activation of which results in potentiation of the histamine secretory response to antigen (Ramkuman et al., J. Biol. Chem. 268:16887-16890, 1993) is evidence in the rat of the role of these receptors in that animal. However, because of the reportedly different pharmacology of the rat A3 receptor as opposed to that of the human and sheep A3 receptors, it could not be predicted that the A3 receptor was significant in primates including humans.

The instant patent disclosure provides evidence that the blockade of A3 adenosine receptor mediated action in the vasculature is useful to treat and prevent disease states in humans.reports that adenosine potentiates the release of granule contents from mast cells isolated from rat peritoneum (Lohse et al., N.-S. Arch. Pharmacol. 335:555-560, 1987; Marquardt et al., J. Immunol. 120:871-878, 1978), and that mast cell degranulation causes constriction in some vascular beds resulting in C5a-induced myocardial ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl.

578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem. 268:16887-16890, 1993). Accordingly, if a method were invented to specifically inhibit mast cell degranulation, a treatment or preventative method would be demonstrated for all of the above known and many yet to be defined disease states associated with adenosine induced mast cell degranulation.

The trigger for mast cell degranulation is usually thought to be an allergen. Allergens are endocytosed by marcrophages and degraded. The resulting fragments are displayed on T lymphocytes. B lymphocytes are stimulated to mature into plasma cells which are able to secrete allergen-specific molecules known as immunoglobulin E, (IgE). These antibodies attach to receptors on mast cells in tissue and on basophils circulating in blood - to trigger degranulation (see L. Lichtenstein, Sci. Am. 269:116-125, 1993). As described below, our data shows that activation of A3 adenosine receptors can produce mast cell degranulation and enhance the effect of allergens. Adenosine and antigens trigger an influx of calcium to induce mast cell granules to release their contents an promote synthesis and release of cytokines, prostaglandins and leukotrienes. The various chemicals released by mast cells are responsible for many allergic symptoms. Long term release of these chemicals can induce basophils, eosinophils, and other cells flowing through blood vessels to migrate into the tissue. Migration is promoted due to the expression and activation of adhesion molecules on the circulating cells and on vascular endotheilial cells. The circulating cells adhere to the endothelial cells, roll among them, and eventually cross into the surrounding matrix. These recruited cells secrete chemicals of their own that damage tissue. Thus, there are long term secondary effects which may also be prevented by specific blockade of mast cell degranulation.

We have shown that specific blockade of the A3 subtype of the adenosine receptor is effective to block the vasoconstrictive response induced through adenosine activation of this receptor subtype. We have also shown that use of a specific inhibitor of the A3 adenosine receptor

subtype is effective to inhibit effects induced by adenosine mediated mast cell degranulation, and have therefore shown that disease states including but not limited to myocardial ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl. 578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem. 268:16887-16890, 1993), are all amenable to prevention and treatment by contacting A3 receptor bearing mast cells with an amount of a selective A3 inhibitor effective to prevent mast cell degranulation.

In another embodiment of this invention, the method is directed to prevention or treatment of myocardial ischemia/reperfusion. The basis of this application of the method is that a period of myocardial ischemia followed by reperfusion produces damage to the myocardium. Part of this damage may be secondary to mast cell degranulation triggered by adenosine during ischemia. This suggests that A3 adenosine receptor antagonists may be useful for the treatment of patients prone to reperfusion injury. This includes patients with coronary artery diseases in general, and patients about to have occluded arteries opened (reperfused) by various interventions (coronary artery bypass grafts, angioplasty or thrombolytic therapy). Adenosine-induced mast cell degranulation during a period of transient ischemia may be responsible for the phenomenon of preconditioning (i.e. a transient ischemic episode reduces myocardial damage resulting from a subsequent prolonged ischemic episode). Accordingly, mast cells are temporarily depleted of damaging mediators during the preconditioning period. These observations are supported by reports suggesting that mast cell degranulation is involved in ischemia/reperfusion injury (see for example Heller, L.J. and Regal, J.F., Effect of adenosine on histamine release and atrioventricular conduction during guinea pig cardia anaphylaxis. Circ. Res. 62:1147-1158, 1988. Conclusion: increases in levels of endogenous adenosine during cardiac anaphylaxis contributed to the development of atrioventricular conduction delays

and that increases in levels of adenosine before antigen challenge may increase the amount of histamine released during cardiac anaphylactic reactions; Wolff, A.A. and Levi, R., Ventricular arrhythmias parallel cardiac histamine efflex after coronary artery occlusion in the dog. Agents and Actions 25:296-306, 1988. Conclusion: During acute myocardial ischemia, the coronary sinus histamine concentration increases simultaneously with the development of early ischemic ventricular arrhythmias and in proportion of their severity; Keller, A.M. Clancy, R.M., Barr, M.L. Marboe, C.C. and Cannon, P.J., Acute reoxygenation injury in the isolated rat heart: role of resident cardiac mast cells. Circ. Res. 63:1044-1052, 1988. conclusion:; The isolated crystalloid-perfused rat heart is not a leukocyte-free preparation and mast cells resident to the heart play an important role in acute reoxygenation injury; Jolly, S.R., Abrams, G.D., Romson, J.L., Bailie, M.B. and Lucchesi, B.R., Effects of lodoxamide on ischemic reperfused myocardium. J. Cardiovas. Pharmacol. 4:441-448, 1982. conclusion: Lodoxamide, a drug that acts to inhibit mast cells degranulation, reduces myocardial ischemic injury; Ito, B.R., Engler, R.L., Del Balzo, U., Role of cardiac mast cells in complement C5a-induced myocardial ischemia. Am. J. Physiol. 33:H1346-H1354, 1992. conclusion: Cardiac mast cells are involved in complement-induced release of vasoactive eicosanoids, including TxA2.).

The human A1, A2a, A2b and A3 receptor subtype cDNAs were subcloned into the expression vectors pSVL (PHARMACIA), CMV5 (Mumby, et al. 1990, PNAS, 87:728-732) or pREP (INVITROGEN). Transient expression in COS7 cells (monkey kidney cell line, ATCC CRL 1651, ATCC, Rockville, MD) was accomplished by transfection of the cloned adenosine receptor cDNAs under the control of the SV40 promoter into mammalian cells (e.g.,COS7). Membranes prepared from the transfected cells were utilized for the determination of binding affinity, selectivity and specificity of the human adenosine receptors for various ligands. Stable expression of the human adenosine receptors in mammalian cells (e.g., CHO, HEK 293) was achieved after integration of the transfected cDNA into the

chromosomes of the host cells. These stable cell lines constituently express the cloned human adenosine receptors and can be propagated infinitely. Stable cell lines expressing the human adenosine subtype cDNAs individually can be used in the binding assay to measure the affinity and selectivity of the receptors for adenosine agonists, antagonists and enhancers.

Membranes prepared from transfected COS7 cells were utilized in a binding assay to measure the affinity of the human adenosine receptors for the radiolabeled adenosine agonists, [^{3}H]-cyclohexyladenosine (CHA), [^{3}H]-CGS21680 (2-(p-(2-carboxyethyl)-phenylamino)-5'-N-ethyl-carboxamidoadenosine), [^{3}H]-5'-N-ethylcarboxamido adenosine ([^{3}H]-NECA), or [^{125}I]-N^6-aminobenzyl adenosine (^{125}I-ABA). Monolayer cell culture of transfected COS7 cells were dissociated with 1 mM EDTA in phosphate buffered saline and resuspended in 5 mM Tris, pH7.6/10 mM $MgCl_2$. The cells were subjected to freeze-thaw lysis and the suspension was homogenized in a glass dounce homogenizer. The membranes were pelleted, resuspended in binding buffer, 50 mM Tris pH 7.6/10 mM $MgCl_2$ and incubated with adenosine deaminase before the binding assay. The binding assay was performed by incubating 50-100 µg of membranes with increasing concentrations of radiolabeled adenosine agonists. Bound ligand was separated from free ligand by filtration on a SKATRON CELL HARVESTER equipped with a receptor binding filtermat. Bound radioactivity was measured by scintillation counting. Substances which bind to or enhance binding to expressed human adenosine receptors in COS and CHO cells can be identified in competition binding assays with radiolabeled adenosine or xanthine analogs. For the competition binding assay, membranes were incubated with 5nM [^{3}H]-CHA 5nM [^{3}H]-CGS21680 or 10nM [^{3}H]-NECA and various concentrations of adenosine agonists or antagonists.

A transient expression system in Xenopus oocytes was established by microinjection of in vitro transcribed mRNA from the cloned adenosine receptor cDNAs. The expression system allows the measurement of the biological effects (i.e., changes in cAMP levels)

upon activation of the expressed adenosine receptors with ligand binding. The cAMP levels are measured by a radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. Activation of the expressed receptors by ligand binding are coupled to either increases or decreases in the intracellular cAMP levels dependent upon the subtype of adenosine receptor (Van Calker et al., (1979) J. Neurochem. 33:999-1003; Londos et al. (1980) Proc. Natl. Acad. Sci. USA 77:2551-2554). The activity of any potential adenosine receptor agonist can be evaluated by measuring the changes in cAMP levels in oocytes injected with adenosine receptor mRNA but not in uninjected or negative control injected oocytes. The activity of any potential adenosine receptor antagonist can be evaluated by determining the inhibition of the cAMP response induced by adenosine in oocytes injected with adenosine receptor transcripts but not negative control or uninjected oocytes. The changes in cAMP accumulation can alternatively be measured in stably transfected CHO cells expressing the human adenosine receptor subtypes.

The cAMP accumulation assay has a number of advantages over the binding assay established in the mammalian cell expression system as a screen for adenosine receptor modulating agents. The assay allows the measurement of a biological effect (i.e., changes in cAMP levels) resulting from the activation of the expressed receptors by ligand binding. The native agonist adenosine is utilized in the assay to activate the expressed receptors. The functionality of additional adenosine receptor subtypes identified by molecular cloning which may not have defined ligands for binding analysis can be evaluated with the natural agonist and without prior identification of a selective, high affinity, radiolabeled ligand.

According to the method of this invention, an adenosine A3 specific antagonist is administered in an amount effective to induce blockade of the receptor. The higher the affinity of the antagonist for the receptor, the lower the required dosage. Compounds having a pKi of greater than about 7 for the A3 receptor and below about 6 for other adenosine receptor subtypes. may be administered by any effective

means to achieve either localized or systemic contact of the antagonist with target A3 adenosine receptors. This might include intraveous, intramuscular, intrasynovial, intranasal, nebulized intrapulmanory, intraperitoneal or other common means for administration of therapeutic compounds. Dosages of between about 1 μg/kg and 10 mg/kg are envisioned, as necessary, to achieve the desired effect of A3 adenosine receptor blockade.

The following examples are provided to further define but not to limit the invention defined by the foregoing description and the claims which follow:

EXAMPLE 1

STEP A:

In the first step of obtaining the partial cDNAs encoding the human A1 and A2a adenosine receptors, total RNA was extracted by homogenizing 2.3g human ventricle in 20 ml 5M guanidine isothiocyanate, 0.1M sodium citrate, pH 6.3, 1mM EDTA, pH 7.0, 5% beta-mercaptoethanol, and 0.5% sodium lauryl sarcosinate. The homogenate was centrifuged for 10 min. at 10,000 rpm and the resulting supernatant was layered onto a cushion of 5.7M CsCl/0.1M EDTA, pH 7.0. After 20 hrs. of centrifugation at 24,000 rpm, the resulting pellet was precipitated one time and then passed over an oligo(dT)-cellulose (PHARMACIA, Piscataway, NJ) column to isolate poly(A)+ RNA.

An oligo(dT) primed library was synthesized from 5 μg of the poly(A)$^+$ human ventricle RNA using the YOU-PRIME cDNA SYNTHESIS KIT (PHARMACIA, Piscataway, NJ). See Gubler and Hoffman Gene 25:263 (1983). The resulting double-stranded cDNA was ligated into λgt10 EcoRI arms (PROMEGA, Madison, WI) and packaged according to the GIGAPACK II GOLD PACKAGING EXTRACT protocol (STRATAGENE, La Jolla, CA). See Huynh et al. (1985) DNA Cloning Techniques: A Practical Approach, IRL Press, Oxford, p.49 and Kretz et al. Res. 17:5409.

The E. coli strain C600Hfl (PROMEGA, Madison, WI) was infected with library phage, plated on agar plates, and incubated at 37°C. The phage DNA was transferred to HYBOND-N nylon membranes (AMERSHAM, Arlington Heights, IL) according to the manufacturer's specifications.

Synthetic probes were constructed from overlapping oligonucleotides (A1 probe: 62+63, A2 probe: 52+53; see Table I for their sequences) based on the published dog A1 (RDC7) and A2a(RDC8) sequences (F Libert, et al,(1989) Science 244:569-572). The oligonucleotides were annealed and filled-in with α^{32}P-dCTP (NEN, Wilmington, DE) and Klenow enzyme. The filters were hybridized with the appropriate probe in 5XSSC, 30% formamide, 5XDenhardt's solution, 0.1% SDS, and 0.1mg/ml sonicated salmon sperm DNA at 42°C, overnight. Following hybridization the filters were washed to a final stringency of 6XSSC at 50°C and exposed to X-OMAT AR film (KODAK, Rochester, NY) at -70°C. The resulting positives were plaque purified by two additional rounds of plating and hybridization. Insert DNA was excised with NotI and ligated into NotI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). (Genebank # 52327) DNA sequences were determined by the SEQUENASE protocol (USBC, Cleveland, OH). See Tabor and Richardsaon, J. Biol. Chem. 264 pp 6447-6458. Two clones were isolated in these screens. The human ventricle A1 cDNA (hva1-3a) and human ventricle A2a cDNA (hva2-13) contain portions of coding sequences for proteins homologous to the reported dog A1 and A2a cDNAs, respectively. The coding region of the human A1 clone corresponds to nucleotides 482 through 981 (Figures 2A and 2B) and is 92% identical to the dog A1 sequence at the nucleotide level. The coding region of the human A2a clone corresponds to nucleotides 497 through 1239 (Figure 4A and 4B), and is 90% identical to the dog A2a sequence at the nucleotide level.

STEP B:

The human ventricle A1 adenosine receptor partial cDNA (hvA1-3a) is a 543 bp NotI fragment containing 23 bp 3' untranslated sequence and is 460 bp short of the initiation methionine based on sequence homology to the dog A1 cDNA. A modification of the 5' RACE (rapid amplification of cDNA ends) method (MA Frohman et al,(1988), Proc. Natl. Acad. Sci. USA, 85:8998-9002) was used to generate the 5' coding region of the cDNA. First strand cDNA was synthesized from 1µg of the human ventricle poly(A)$^+$ RNA in a total volume of 40µl containing 50mM Tris, pH 8.0, 140mM KCl, 10mM $MgCl_2$, 10mM DTT, 15mM each dNTP, 20 units RNasin (PROMEGA, Madison, WI), 20pmol human primer 79 (see Table I), and 9.2 units AMV reverse transcriptase at 37°C for 2 hrs. The reaction was then diluted to 120 ml with 0.5 mM Tris, pH 7.6/0.05 mM EDTA and passed through a SEPHACRYL S-300 SPUN COLUMN (PHARMACIA, Piscataway, NJ). The product in the column effluent was polyadenylated in 100mM potassium cacodylate, pH 7.2, 2mM $CoCl_2$, 0.2mM DTT, 0.15mM dATP, and 14 units terminal deoxynucleotidyl transferase in a total volume of 31µl for 10 min. at 37°C. The reaction was terminated by heating at 65°C for 15 min. and then diluted to 500 µl with 10 mM Tris, pH 8.0/1 mM EDTA (TE).

Ten µl of the poly(A)-tailed first strand cDNA was used as template in a primary PCR amplification reaction according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT; see Saiki et al. (1988) Science 239:487-491) containing 10pmol primer 70, 25pmol primer 71, and 25pmol human primer 80 (see table I) in a total volume of 50 µl. Primer 70 is 5'-gactcgagtcgacatcga$(t)_{17}$, primer 71 is 5'-gactcgagtcgacatcga, and both are based on MA Frohman, et al (1988), Proc. Natl. Acad. Sci. USA, 85:8998-9002. One cycle of PCR was performed of 1 min at 95°C, 2 min at 50°C, 40 min at 72°C, followed by 40 cycles of 40 sec at 94°C, 2 min at 56°C, 3 min at 72°C. The primary PCR amplification reaction product was electrophoresed through a 1.4% agarose gel and an area corresponding to approximately 600 bp was excised. The gel slice was melted and 1 µl was used as

template in a secondary PCR amplification reaction containing 100pmol primer 71 and human primer 81 (see Table I) for 30 cycles of 1 min at 94°C, 2 min at 56°C, 3 min at 72°C. The secondary PCR amplification product was digested with EcoRI and SalI and electrophoresed on a 1.4% agarose gel. An area corresponding to 500-600bp was excised and ligated into EcoRI/SalI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequence of the 515 bp PCR product (5'HVA1-9) was determined by the SEQUENASE protocol (USBC, Cleveland, OH). The partial human ventricle A1 cDNA and the PCR product contain overlapping sequence and represent the complete coding region for the human A1 receptor, including 14 and 23 bp of 5' and 3' untranslated sequences, respectively. The sequence of the human A1 adenosine receptor cDNA so identified, is shown in Figures 2A and 2B.

STEP C:

A probe was generated by Klenow enzyme extension, including α^{32}P-dCTP, of annealed oligonucleotides 62 and 63, and used to screen a human kidney cDNA library (CLONTECH, Palo Alto, CA). E. coli strain C600hfl (PROMEGA, Madison, WI) was infected with library phage and grown overnight on agar plates at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750mM NaCl, 75mM sodium citrate, 30% formamide, 0.1% sodium dodecyl sulfate, 0.5mg/mL polyvinylpyrrolidone, 0.5mg/mL bovine serum albumin, 0.5mg/mL Ficoll 400, and 0.1mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9M NaCl and 90mM sodium citrate at 50°C. A positively hybridizing phage (hkA1-14), was identified and purified by replating and screening with the probe twice more. The final phage plaque was transferred to 0.5 mL 50mM Tris, pH 7.5, 8mM $MgSO_4$, 85 mM NaCl, 1mg/mL gelatin, and 1 µL of a 1:50 dilution in water of the phage stock was used as template for PCR amplification. 50 pmol each of 1amL and 1amR (Table I) oligonucleotide primers were included,

then a final 15 min at 72°, according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT). A 2.0 kb product was identified by agarose gel electrophoresis, and this was subcloned into the EcoRI site of pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). Sequence analysis by the SEQUENASE protocol (USBC, Cleveland, OH) demonstrated that this cDNA was homologous to the reported dog A1 clone. SmaI and EcoRI digestion released a DNA fragment containing coding sequence from base pair 76 through the translation STOP codon (Figure 2) that is identical to the human ventricle A1 cDNA sequence (clones hva1-3a and 5'hva1-9). This fragment was used in construction of the full length coding sequence (see below). The human kidney cDNA also includes about 900 bp of 3' untranslated sequence.

STEP D:

The human ventricle A2a adenosine receptor partial cDNA (hvA2-13) is a 1.6 kb NotI fragment containing approximately 900 bp 3' untranslated sequence and is 496 bp short of the initiation methionine based on sequence homology to the dog A2a cDNA clone. Two consecutive rounds of 5' RACE were utilized to generate the 5' coding region of the cDNA. First strand cDNA was synthesized from 1 μg of the human ventricle poly(A)$^+$ RNA in a total volume of 40 μl containing 50mM Tris, pH 8.0, 140mM KCl, 10mM $MgCl_2$, 10mM DTT, 15mM each dNTP, 20 units RNasin, 20pmol human primer 68 or 74 (for 1st or 2nd round RACE respectively), and 9.2 units AMV reverse transcriptase at 37°C for 2 hrs. The reaction was then diluted to 120μl with 0.5 mM Tris, pH 7.6/0.05 mM EDTA and passed through a SEPHACRYL S-300 SPUN COLUMN. The products in the column effluents were polyadenylated in 100mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM DTT, 0.15 mM dATP, and 14 units terminal deoxynucleotidyl transferase in a total volume of 31 μl for 10 min. at 37°C. The poly(A) tailing reaction was terminated by heating at 65°C for 15 min. and then diluted to 500 μl with TE.

Five or 10 µl (for 1st or 2nd round RACE respectively) of the poly(A) tailed first strand cDNA was used as template in the PCR amplification reaction according to the GENEAMP protocol containing 10pmol primer 70, 25 pmol primer 71 (primer 70 and 71 sequences are given above), and 25 pmol human primer 69 or 75 (1st or 2nd round RACE respectively; see Table I) in a total volume of 50 µl. One cycle of PCR was performed of 1 min at 95°C, 2 min at 50°C, 40 min at 72°C, followed by 40 cycles of 40 sec at 94°C, 2 min at 56°C, 3 min at 72°C. The PCR amplification products were digested with EcoRI and SalI and electrophoresed on a 1.4% agarose gel. Areas corresponding to 200-400 bp were excised and ligated into EcoRI/SalI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequences of the two A2a PCR products, the 332 bp 1st round RACE product (5'hvA2-14) and the 275 bp 2nd round RACE product (5'hvA2-29) were determined by the SEQUENASE (USBC, Cleveland, OH) protocol. By sequence homology comparisons with the dog A2a adenosine receptor cDNA sequence, the 1st round RACE product (5'hvA2-14) was 258 bp short of the initiation methionine and the second round RACE product (5'HVA2-29) was determined to extend 1bp upstream of the initiation methionine. The human ventricle A2a partial cDNA clone (hvA2-13) and the human A2a PCR products (5'hvA2-14 and 5'hva2-29) contain overlapping sequence and together represent the complete coding sequence for the human adenosine A2a receptor, and include 1 bp and 0.8 kb of 5' and 3' untranslated sequence, respectively. The sequence of the human A2a adenosine receptor is shown in Figures 4A and 4B.

STEP E:

A double-stranded DNA probe was generated by Klenow enzyme extension, including α^{32}P-dCTP, of annealed oligonucleotides 66 and 67, and used to screen a human striata cDNA library (STRATAGENE, La Jolla, CA). The oligonucleotide sequence was based on a region of the human ventricle A2a cDNA sequence. E. coli strain XL1-blue (STRATAGENE, La Jolla, CA) cells were infected with library phage and grown overnight on agar plates at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750 mM NaCl, 75 mM sodium citrate, 10% formamide, 0.5% sodium dodecyl sulfate, 0.5 mg/mL polyvinylpyrrolidone, 0.5 mg/mL bovine serum albumin, 0.5 mg/mL Ficoll 400, and 0.02 mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9 M NaCl and 90 mM sodium citrate at 50°C. A positively hybridizing phage (hbA2-22A) was identified and purified by replating and screening with the probe twice more, and subcloned into the plasmid pBLUESCRIPT SK- by the manufacturer's protocol (STRATAGENE, La Jolla, CA). See Short et al. (1988) Nucl. Acids Res. 16:7583-7600; Sorge (1988) Stratagies 1:3-7. The human brain A2a adenosine receptor cDNA (hbA2-22A) spans bp 43 of the A2 coding sequence (Figures 4A and 4B) through the translation STOP codon, and includes about 900 bp of 3' untranslated sequence. The sequence of this human brain A2a cDNA is identical to the human ventricle A2a adenosine receptor cDNA (hvA2-13, 5'hvA2-14 and 5'hvA2-29).

STEP F:

A double-stranded DNA probe was generated by Klenow enzyme extension of annealed oligonucleotides 129 and 130, including α^{32}P-dCTP, and used to screen a human frontal cortex cDNA library (STRATAGENE, La Jolla, CA). The oligonucleotide sequence was based on a region of the human A2a and A1 cDNA sequence. E. coli strain XL-1 blue (STRATAGENE, La Jolla, CA) cells were infected

transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750 mM NaCl, 75 mM sodium citrate, 10% formamide, 0.5% sodium dodecyl sulfate, 0.5 mg/mL polyvinyl-pyrrolidone, 0.5 mg/mL bovine serum albumin, 0.5 mg/mL Ficoll 400, and 0.02 mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9 M NaCl and 90 mM sodium citrate at 50°C. A positively hybridizing phage (hb-32c), was identified and purified by replating and screening with the probe twice more. The insert was subcloned to the plasmid pBLUESCRIPT SK- according to the manufacturer's protocol (STRATAGENE, La Jolla, CA). Sequence analysis by the SEQUENASE protocol (USBC, Cleveland, OH) demonstrated a complete open reading frame coding for amino acid sequence homologous to both of the previously isolated human A1 and A2a clones. This homologous adenosine receptor subtype cDNA is the A2b subtype having the sequences in Figures 5 and 6. A 1.3 kb SmaI-XmnI fragment was ligated into the SmaI site of pSVL (PHARMACIA, Piscataway, NJ), giving the full length coding sequence of the A2b adenosine receptor in a plasmid suitable for its expression in COS and CHO cells. See Sprague et al. (1983) J. Virology 45:773; Templeton and Eckhart (1984) Mol. Cell Biol. 4:817.

Table I:

Sequences and directions of the primers used in the isolation of cDNA's and construction of expression plasmids, along with the positions in the clones upon which the sequences are based. Dog A1 and A2a cDNA clones are from F. Libert, et al, (1989) Science 244:569-572. Primers LamL and LamR are based on the sequence of λgt10 (T.V. Hyunh, et al. (1985) DNA Cloning: A Practical Approach, Vol 1, D. Glover, ed, IRL Press, Oxford). The A2b adenosine receptor subtype encoded by the clone hb32C was determined to be the A2b adenosine receptor subtype on the basis of the binding profile of the adenosine receptor agonist NECA and affinities for adenosine receptor

antagonists measured on membranes prepared from pSVLhb32C transfected COS7, CHO or HEK 293 cells.

name	sequence	position	clone	direction
52	ATTCGCAGCCACGTCCTGA-GGCGGCGGGAGCCCTTCAA-AGCAGGTGGCACCAGTGCC-CGC (SEQ ID NO. 1)	1201-1260	dog A2a	sense
53	GCGGAGGCTGATCTGCT-CTCCATCACTGCCATGAG-CTGCCAAGGCGCGGGCAC-TGGTGCC (SEQ. ID NO. 2)	1305-1246	dog A2a	antisense
62	TCCAGAAGTTCCGGGTCA-CCTTCCTTAAGATCTGGAA-TGACCACTTCCGCTGCCAGC-CCA (SEQ. ID NO. 3)	958-1017	dog A1	sense
63	AGTCGTGGGGCGCCTCCT-CTGGGGGGTCCTCGTCGAC-GGGGGGCGTGGGCTGGCAG-CGGA (SEQ ID NO. 4)	1062-1003	dog A1	antisense
66	GCCTCTTTGAGGATGTGG-TCCCCATGAACTACATGGT-GTACTTCA (SEQ ID NO. 5)	500-542	5'hvA2-14	sense
67	GCAGGGGCACCAGCACACA-GGCAAAGAAGTTGAAGTAC-ACCATGT (SEQ ID NO. 6)	572-528	5'hva2-14	antisense

name	sequence	position	clone	direction
68	TCGCGCCGCCAGGAAGAT (SEQ ID NO 7)	616-599	hva2-13	antisense
69	TATATTGAATTCTAGACAC-CCAGCATGAGC (SEQ ID NO.8)	591-574	hva2-13	antisense
74	TCAATGGCGATGGCCAGG (SEQ ID NO. 9)	303-286	5'hva2-14	antisense
75	TATATTGAATTCATGGA-GCTCTGCGTGAGG- (SEQ ID NO. 10)	276-259	5'hva2-14	antisense
79	GTAGACCATGTACTCCAT (SEQ ID NO. 11)	560-543	hva1-3a	antisense
80	TATATTGAATTCTGACCT-TCTCGAACTCGC- (SEQ ID NO. 12)	537-521	hva1-3a	antisense
81	ATTGAATTCGATCACGGG-CTCCCCCATGC- (SEQ ID NO. 13)	515-496	hva1-3a	antisense
129	ATGGAGTACATGGTCTAC-TTCAACTTCTTTGTGTGGG-TGCTGCCCCCGCT- (SEQ ID NO. 14)	---	---	sense

name	sequence	position	clone	direction
130	GAAGATCCGCAAATAGACA-CCCAGCATGAGCAGAAGCG-GGGGCAGCACCC (SEQ ID NO. 15)	---	---	antisense
131	CCCTCTAGAGCCCAGCCTGT-GCCCGCCATGCCCATCATGG-GCTCC (SEQ ID NO. 16)	2-19 1-14	5'hva2-29 5'hva1-9	sense
lamL	CCCACCTTTTGAGCAAGTTC (SEQ ID NO. 17)	---	λt10	---
lamR	GGCTTATGAGTATTTCTTCC (SEQ ID NO. 18)	---	λt10	---
207	CCCAAGCTTATGAAAGCCAA CAATACC (SEQ ID NO. 27)			
208	TGCTCTAGACTCTGGTATCT TCACATT (SEQ ID NO. 28)			

EXAMPLE 2

Human A1 adenosine receptor expression construct:

To express the human adenosine receptor cDNA in COS, CHO and HEK 293 cells, the 118bp SalI-SmaI fragment of the human ventricle A1 PCR product (5'HVA1-9) was ligated together with the 1.8 SmaI-EcoRI fragment of the human kidney A1 adenosine receptor cDNA (hkA1-14) and the 3.0 kb SalI-EcoRI fragment of

pBLUESCRIPT II KS+, resulting in a plasmid containing the contiguous full length coding sequence for the human A1 adenosine receptor cDNA and some 5' and 3' untranslated sequence. This plasmid was digested first with EcoRI, the resulting ends were filled in by Klenow enzyme extension and then the plasmid was digested with XhoI to release a fragment of 1.9 kb containing the full length human A1 adenosine receptor cDNA. The fragment was subcloned into the expression vector pSVL (PHARMACIA) which had been digested with XhoI-SmaI.

Human A2a adenosine receptor expression construct:

To express the human A2a adenosine receptor cDNA in COS, CHO or HEK 293 cells, a contiguous A2a cDNA sequence was constructed before subcloning into the expression vector, pSVL. Primer 131, containing an XbaI recognition site, 14 bp of 5' untranslated sequence of human A1 adenosine receptor cDNA, and the first 18 bp of human A2a adenosine receptor cDNA coding sequence was used with primer 75 in PCR with 1 ng of the plasmid containing the human ventricle A2a 2nd round RACE product (5'hvA2-29) as template. Twenty-five cycles of 40 sec at 94°C, 1 min at 55°C, and 3 min at 72°C, then a final incubation of 15 min at 72°C, with 1 ng of plasmid template and 50 pmol of each primer in a volume of 50 µL according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT), resulted in the expected 302 bp product determined by agarose gel electrophoresis. The 172 bp XbaI-EagI digestion product of this DNA fragment was ligated together with 1125 bp EagI-BglII digestion product of the human striata A2a adenosine receptor cDNA (hbA2-22A) and XbaI-SmaI digested pSVL (PHARMACIA), generating the full length human A2a adenosine receptor cDNA coding sequence in a plasmid suitable for its expression in COS, CHO or HEK 293 cells.

Mammalian cell expression:

COS7 cells (ATCC #1651-CRL) were grown in complete medium, Dulbecco's modified Eagles's medium, DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 100U/mL

penicillin-streptomycin and 2 mM glutamine, in 5% CO_2 at 37°C. Transient transfection of COS7 cells was performed by the $CaPO_4$ method (Graham,F.L. and Van Der Erb, A.J. (1973) Virology 52:456-567) using the Mammalian Transfection Kit (STRATAGENE). See Chen and Okayama Mol. Cell Biol. 7:2745-2752. Plasmid DNA (15 µg) was precipitated with 125 mM $CaCl_2$ in BBS (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffered saline) at room temperature for 30 minutes. The DNA precipitate was added to the COS7 cells and incubated for 18h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated in complete medium in 5% CO_2 at 37°C for 48 h prior to the binding assay.

Stable expression in CHO or HEK 293 cells:

To establish stable cell lines, CHO or HEK 293 cells were co-transfected with 20 µg of pSVL containing the adenosine receptor cDNA and 1µg of pWLneo (STRATAGENE) containing the neomycin gene. See Southern and Berg (1982) J. Mol. App. Gen. 1:327-341. Transfection was performed by the $CaPO_4$ method. DNA was precipitated at room temperature for 30 minutes, added to the CHO cells and incubated 18h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated for 24h in 5% CO_2 at 37°C, replated in 24-well dishes at a dilution of 1:10, and incubated an additional 24h before adding selection medium, DMEM containing 10% fetal bovine serum, 100U/mL penicllin-streptomycin, 2 mM glutamine and 0.5 mg/mL G418 (GIBCO). Transfected cells were incubated at 5% CO_2, 37°C until viable colonies were visible, approximately 14-21 days. Colonies were selected and propagated. The cell clone with the highest number of human adenosine receptors was selected for subsequent application in the binding assay.

EXAMPLE 3

Binding studies:

Membranes were prepared from transiently transfected COS7 cells 48 h after transfection or from G418-selected stably transfected CHO or HEK 293 cells. Cells were harvested in 1 mM EDTA in phosphate buffered saline and centrifuged at 2000 x g for 10 minutes. The cell pellet was washed once with phosphate buffered saline. The cell pellet was resuspended in 2 mL of 5 mM Tris, pH 7.6/ 5mM $MgCl_2$. Membranes were prepared from the cells by freeze-thaw lysis in which the suspension was frozen in a dry ice/ethanol bath and thawed at 25°C twice. The suspension was homogenized after adding an additional 2 mL of 5 mM Tris, pH 7.6/5 mM $MgCl_2$, in a glass dounce homogenizer with 20 strokes. The membranes were pelleted at 40,000 x g at 4°C for 20 minutes. The membrane pellet was resuspended at a protein concentration of 1-2 mg/mL in binding assay buffer, 50 mM Tris, pH 7.6/10 mM $MgCl_2$. Protein concentration was determined by the method of Bradford ((1976) Anal. Biochem. 72: 248-250). Before the binding assay was performed, the membranes were incubated with adenosine deaminase (BOEHRINGER MANNHEIM), 2 U/mL for 30 minutes at 37°C. Saturation binding of [^{3}H]-cyclohexyladenosine (CHA) was performed on membranes prepared from pSVLA1 transfected COS7 or CHO cells.

Membranes (100µg) were incubated in the presence of 0.2 U/mL adenosine deaminase with increasing concentrations of CHA (NEN, 32 Ci/mmol) in the range of 0.62 - 30 nM for 120 minutes at 25°C in a total volume of 500 µL. The binding assay was terminated by rapid filtration and three washes with ice-cold 50 mM Tris,pH 7.6/10 mM $MgCl_2$ on a SKATRON CELL HARVESTER equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined in the presence of 100 µM N^6-cyclopentyladenosine (CPA). Bound radioactivity was measured by scintillation counting in READY SAFE SCINTILLATION COCKTAIL (BECKMAN). For competition binding experiments, membranes were

incubated with 5 nM [^{3}H]-CHA and various concentrations of A1 adenosine receptor agonists. Saturation binding of [^{3}H] CGS-21680 was performed on membranes prepared from pSVLA2a transfected COS7 cells. Membranes (100μg) were incubated in the presence of 0.2 U/mL adenosine deaminase with increasing concentrations of CGS21680 (NEN, 48.6 Ci/mmol) in the range of 0.62 -80 nM for 90 minutes at 25°C in a total volume of 500 μL. The binding assay was terminated by rapid filtration with three washes with ice-cold 50 mM Tris, pH 7.6/10 mM $MgCl_2$ on a Skatron cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined in the presence of 100μM CPA. Bound radioactivity was measured by scintillation counting in READY SAFE LIQUID SCINTILLATION COCKTAIL (BECKMAN). For competition binding experiments, membranes were incubated with 5nM [^{3}H]-CGS21680 and various concentrations of A2 adenosine receptor agonists.

Saturation binding of [^{3}H]5'-N-ethylcarboxamidoadenosine (NECA) was performed on membranes (100 μg) prepared from pSVLhb32C (A2b) transfected COS7 cells in the presence of adenosine deaminase with increasing concentrations of NECA (NEN, 15.1Ci/mmol) in the range of 1.3-106 nM for 90 minutes at 25°C in a total volume of 500 μL. The assay was terminated by rapid filtration and three washes with ice-cold binding buffer on a cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Bound radioactivity was measured by scintillation counting. Non-specific binding was measured on membranes prepared from non-transfected COS7 cells. For competition binding experiments, membranes from transfected cells were incubated with 10 nM [^{3}H]NECA and varying concentrations of adenosine receptor antagonists.

EXAMPLE 4

The human A3 adenosine receptor was cloned from a human striata cDNA library. Oligonucleotide probes were designed based on the rat A3 sequence of Zhou et al., Proc. Natl. Acad. Sci. 89, 7432 (1992). The complete sequence of the human A3 adenosine receptor was determined and the protein sequence deduced. The cloned human A3 adenosine receptor is expressed in a heterologous expression system in COS, CHO and HEK 293 cells. Radiolabeled adenosine receptor agonists and antagonists are used to measure the binding properties of the expressed receptor. Stable cell lines can be used to evaluate and identify adenosine receptor agonists, antagonists and enhancers.

STEP A:

A synthetic probe homologous to the rat A3 adenosine receptor was generated using the polymerase chain reaction (PCR). Three µl of rat brain cDNA was used as template in a PCR amplification reaction according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT) containing 50 pmol of primers 207 (5'-cccaagcttatgaaagccaacaatacc) (SEQ. ID NO: 27) and 208 (5'-tgctctagactctggtatcttcacatt) (SEQ. ID NO: 28) in a total volume of 50 µl. Primers 207 and 208 are based on the published rat A3 adenosine receptor sequence (Zhou, et al, (1992), Proc. Natl. Acad. Sci. USA, 89:7432-7406). Forty cycles of 40 sec at 94°C, 1 min at 55°C, 3 min at 72°C were performed and the resulting 788 bp fragment was subcloned into HindIII-XbaI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequence was verified by the SEQUENASE protocol (USBC, Cleveland, OH).

STEP B:

The 788 bp PCR fragment was labeled with α^{32}P-dCTP using the MULTIPRIME DNA LABELLING SYSTEM (AMERSHAM, Arlington Heights, IL) and used to screen a human striata cDNA library

(STRATAGENE, La Jolla, CA). E. coli strain XL-1 Blue (STRATAGENE, La Jolla, CA) cells were infected with library phage and grown overnight at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 5 X SSC, 30% formamide, 5 X Denhardt's solution, 0.5% sodium dodecyl sulfate, and 50 μg/ml sonicated salmon testis DNA. The filters were washed in 2 X SSC at 55°C. A positively hybridizing phage (HS-21a) was identified and plaque purified by two additional rounds of plating and hybridization. The insert was subcloned to the plasmid pBLUESCRIPT II SK- according to the manufacturer's protocol (STRATAGENE, La Jolla, CA). Upon sequence analysis using the SEQUENASE protocol (USBC, Cleveland, OH) it was determined that clone HS-21a contained the complete open reading frame corresponding to the human homolog of the rat A3 adenosine receptor. The coding region of the human A3 adenosine receptor cDNA is 78% identical to the rat sequence at the nucleotide level and contains 265 bp and 517 bp of 5' and 3' untranslated sequence, respectively. The 1.7 kb fragment was excised using sites present in the multiple cloning site of pBLUESCRIPT II SK- (STRATAGENE, La Jolla, CA) and subcloned into XhoI/SacI digested pSVL (PHARMACIA, Piscataway, NJ) for its expression in COS and CHO cells.

EXAMPLE 5

Mammalian cell expression:

COS7 cells (ATCC #1651-CRL) were grown in complete medium, Dulbecco's modified Eagles's medium, DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin and 2mM glutamine, in 5% CO_2 at 37°C. Transient transfection of COS7 cells was performed by the $CaPO_4$ method (Graham, F.L. and Van Der Erb, A.J. (1973) Virology 52:456-567) using the Mammalian Transfection Kit (STRATAGENE). Plasmid DNA (15 μg) was precipitated with 125 mM $CaCl_2$ in BBS (N,N-bis(2-

hydroxyethyl)-2-aminoethanesulfonic acid buffered saline) at room temperature for 30 minutes. The DNA precipitate was added to the COS7 cells and incubated for 18 h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated in complete medium in 5% CO_2 at 37°C for 48 h prior to the binding assay.

Stable expression in CHO cells:

To establish stable cell lines, CHO cells were cotransfected with 20 µg of pSVL containing the adenosine receptor cDNA and 1 µg of pWLneo (STRATAGENE) containing the neomycin gene. Transfection was performed by the $CaPO_4$ method. DNA was precipitated at room temperature for 30 minutes, added to the COS7 cells and incubated 18 h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated for 24 h in 5% CO_2 at 37°C, replated in 24-well dishes at a dilution of 1:10, and incubated an additional 24 h before adding selection medium, DMEM containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin, 2 mM glutamine and 1.0 mg/mL G418 (GIBCO). Transfected cells were incubated at 5% CO_2, 37°C until viable colonies were visible, approximately 14-21 days. Colonies were selected and propagated. The cell clone with the highest number of human adenosine receptors was selected for subsequent application in the binding assay.

EXAMPLE 6

Binding assay:

Membranes were prepared from transiently transfected COS7 cells 48 h after transfection or from G418-selected stably transfected CHO or HEK 293 cells. Cells were harvested in 1 mM EDTA in phosphate buffered saline and centrifuged at 2000 x g for 10 minutes. The cell pellet was washed once with phosphate buffered saline. The cell pellet was resuspended in 2 mL of 5 mM Tris, pH 7.6/

5mM $MgCl_2$. Membranes were prepared from the cells by freeze-thaw lysis in which the suspension was frozen in a dry ice/ethanol bath and thawed at 25°C twice. The suspension was homogenized after adding an additional 2 mL of 5 mM Tris, pH 7.6/ 5mM $MgCl_2$, in a glass dounce homogenizer with 20 strokes. The membranes were pelleted at 40,000 x g at 4°C for 20 minutes. The membrane pellet was resuspended at a protein concentration of 1-2 mg/mL in binding assay buffer, 50 mM Tris, pH 7.6/10 mM $MgCl_2$. Protein concentration was determined by the method of Bradford ((1976) Anal. Biochem. 72: 248-250). Before the binding assay was performed, the membranes were incubated with adenosine deaminase (BOEHRINGER MANNHEIM), 2U/mL for 30 minutes at 37°C. Saturation binding of [^{125}I]-N^6-aminobenzyl-adenosine (^{125}I-ABA) or [^{125}I]-N^6-2-(4-amino-3-iodophenyl)ethyl-adenosine (APNEA) was performed on membranes prepared from pSVLA3 transfected COS7 cells. Membranes (100 µg) were incubated in the presence of 0.2U/mL adenosine deaminase with increasing concentrations of ^{125}I-ABA in the range of 0.1-30 nM for 120 minutes at 25°C in a total volume of 500 µL. The binding assay was terminated by rapid filtration and three washes with ice-cold 50 mM Tris, pH 7.6/10 mM $MgCl_2$ on a Skatron cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined on non-transfected cells. Bound radioactivity was measured by scintillation counting in Ready Safe Scintillation Cocktail (BECKMAN).

EXAMPLE 7

In vitro transcription and oocyte expression:

The 1.3 kb XhoI-BamHI fragment of the pSVL expression construct (described in Example 2) containing the full length human A2a adenosine receptor coding sequence was ligated into SalI-SpeI digested pGEMA (Swanson, et al, (1990) Neuron 4:929-939). The resulting plasmid, pGEMA2, was linearized with NotI, forming a template for in vitro transcription with T7 RNA polymerase. The

homologous adenosine receptor subtype cDNA in pBluescript SK- was used as a template for in vitro transcription by T3 polymerase after removal of most of the 5' untranslated region, with the exception of 20 bp, as a 0.3 kb SmaI fragment. The K^+ channel cDNA, Kv3.2b was employed as a negative control in the cAMP accumulation assay. The generation of Kv3.2b RNA was described by Luneau, et al, ((1991) FEBS Letters 1:163-167). Linearized plasmid templates were used with the STRATAGENE mCAP kit according to the manufacturer's protocol, except that the SP6 RNA polymerase reaction was performed at 40°C. Oocytes were harvested from mature female *Xenopus laevis*, treated with collagenase, and maintained at 18°C in ND96 medium (GIBCO) supplemented with 1 mM sodium pyruvate and 100 µg/mL gentamycin. Fifty nanoliters (10 ng) of RNA diluted in H_2O was injected and oocytes were incubated at 18°C for 48 hours.

EXAMPLE 8

cAMP accumulation assay in oocytes:

Oocytes injected with either human adenosine receptor transcript or the Kv3.2b transcript were transferred to fresh medium supplemented with 1 mM of the phosphodiesterase inhibitor, Ro 20-1724 (RBI, Natick, MA) and 1 mg/mL bovine serum albumin incubated for 30 minutes and transferred to an identical medium with or without the agonist adenosine (10 mM) for an additional 30 minutes at room temperature. Groups of 5-10 oocytes were lysed by transfer to ND96/100 mM HCl/1 mM Ro 20-1724 in microfuge tubes, shaken, incubated at 95°C for 3 min, and centrifuged at 12000 g for 5 min. Supernatants were stored at -70°C before cAMP measurements. Cyclic AMP levels were determined by radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. The adenosine receptor antagonist, 8-(p-sulfophenyl)theophylline (100 µM) was utilized to inhibit the cAMP response induced by adenosine in oocytes expressing the adenosine receptors.

EXAMPLE 9

cAMP accumulation in stable CHO cell lines:

The changes in cAMP accumulation can alternatively be measured in stably transfected CHO cells expressing the human adenosine receptor subtypes. CHO cells are washed twice in phosphate buffered saline (PBS) and detached in 0.2% EDTA in PBS. The cells are pelleted at 800 rpm for 10 min and resuspended in KRH buffer (140 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM KH_2PO_4/6 mM glucose/25 mM Hepes buffer, pH 7.4). The cells are washed once in KRH buffer and resuspended at 10^7 cells/mL. The cell suspension (100 µL) is mixed with 100 µL of KRH buffer containing 200 µM Ro 20-1724 and incubated at 37°C for 10 minutes. Adenosine (10 µM) was added in 200 µL KRH buffer containing 200 µM Ro 20-1724 and incubated at 37°C for 20 minutes. After the incubation, 400 µL of 0.5 mM NaOAc (pH 6.2) was added and the sample was boiled for 20 minutes. The supernatant was recovered by centrifugation for 15 minutes and stored at -70°C. cAMP levels were determined by radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. The effect of antagonists on cAMP accumulation are measured by preincubation for 20 minutes before adding adenosine.

EXAMPLE 10

Expression Construct and Transfection

The 1.7 kb HS-21a cDNA (A3) was subcloned as a SalI-BamHI fragment into the expression vector pCMV5 (Mumby, S.M., Heukeroth, R.O., Gordon, J.I.and Gilman, A.G. (1990) Proc. Natl. Acad. Sci. USA **87**, 728-732.) creating the vector pCMV5-A3. CHO or HEK 293 cells stably expressing the human HS-21a cDNA were prepared by co-transfection of 15 µg pCMV5-A3 and 1 µg pWLneo (Stratagene) using the calcium phosphate method. Stable cell lines were also generated using EBV based mammalian expression vectors, pREP (INVITROGEN). Neomycin resistant colonies were selected in 1

mg/mL G418 (GIBCO). Stable colonies were screened for expression of HS-21a by ^{125}I-ABA binding.

EXAMPLE 11

Binding Studies

Membranes were prepared from stable CHO cell lines in 10 mM Hepes, pH 7.4 containing 0.1 mM benzamidine and 0.1 mM PMSF as described (Mahan, L.C., et al., (1991) Mol. Pharmacol. **40**, 1-7). Pellets were resuspended in 5 mM Hepes, pH 7.4/5 mM $MgCl_2$/0.1 mM benzamidine/0.1 mM PMSF at a protein concentration of 1-2 mg/mL and were incubated with adenosine deaminase (Boehringer Mannheim), 2U/mL at 37 °C for 20 minutes. Saturation binding of ^{125}I-ABA was carried out on 50 µg of membranes for 120 minutes at 25 °C in a total volume of 100 µL. The assay was terminated by rapid filtration and three washes with ice-cold binding buffer on a Skatron harvester equipped with a receptor binding filtermat (Skatron Instruments, INC). The specific activity of ^{125}I-ABA, initially 2,200 Ci/mmol, was reduced to 100 Ci/mmol with nonradioactive I-ABA for saturation analysis. Nonspecific binding was measured in the presence of 1 µM I-ABA. The K_D and B_{max} values were calculated by the EBDA program (McPherson, G.A. (1983) Computer Programs for Biomedicine **17**, 107-114). Competition binding of agonists and antagonists was determined with ^{125}I-ABA (0.17-2.0 nM, 2000 Ci/mmol). Nonspecific binding was measured in the presence of 400 µM NECA. Binding data were analyzed and competition curves were constructed by use of the nonlinear regression curve fitting program Graph PAD InPlot, Version 3.0 (Graph Pad Software, San Diego). K_i values were calculated using the Cheng-Prusoff derivation (Cheng, Y.C. and Prusoff, H.R. (1973) Biochem. Pharmacol. **22**, 3099-3108.).

The binding properties of the receptor encoded by HS-21a were evaluated on membranes prepared from CHO cells stably expressing the HS-21a cDNA. The radioligand, ^{125}I-APNEA, was previously used to characterize rat A3 adenosine receptors. In preliminary experiments, high non-specific ^{125}I-APNEA binding to

CHO cell membranes was observed which interfered with the measurement of specific binding to expressed receptors. Specific and saturable binding of the adenosine receptor agonist, ^{125}I-ABA was measured on membranes prepared from the stably transfected cells (Figure 11A). The specific binding of ^{125}I-ABA could be prevented by either 1 mM nonradioactive I-ABA or 400 μM NECA. No specific binding of ^{125}I-ABA was measured on membranes prepared from non-transfected CHO cells. The specific binding of ^{125}I-ABA measured in either the presence of 10 μM GTPγS or 100 mM Gpp(NH)p was reduced by 56 and 44% respectively, relative to the specific binding measured in the absence of the uncoupling reagents. These results suggest that ^{125}I-ABA exhibits some agonist activity on the receptor encoded by the HS-21a cDNA expressed in the stable CHO cell line. ^{125}I-ABA binds to membranes prepared from the HS-21a stable CHO cells with a dissociation constant of 10 nM (B_{max}= 258 fmol/mg protein) with a Hill coefficient of 0.99 indicating binding to a single class of high affinity sites (Figure 11B).

The competition of adenosine receptor agonists and antagonists for binding to HS-21a receptors was determined (Figures 12A and 12B). The K_i values for agonists (top panel) were calculated to be 26 nM for NECA, 34 nM for R-PIA, 89 nM for CPA and 320 nM for S-PIA, resulting in a potency order profile of NECA >R-PIA > CPA > S-PIA. In contrast to the insensitivity of adenosine receptor antagonists reported for the rat A3 adenosine receptor subtype, a number of xanthine antagonists exhibited competition with ^{125}I-ABA for binding to the HS-21a receptor (lower panel). Studies of the sheep A3 adenosine receptor indicated that 8-phenylxanthines substituted in the para-position with acidic substituents are high affinity antagonists. By evaluating additional xanthines in this class we determined that I-ABOPX is the highest affinity antagonist yet reported for A3 adenosine receptors. The K_i values for antagonists were calculated to be 18 nM for I-ABOPX, 55 nM for BW-A1433, 70 nM for XAC and 750 nM for DPCPX, resulting in a potency order profile of I-ABOPX >BW-A1433 > XAC >DPCPX.

EXAMPLE 12

cAMP Studies

Determinations were made on stably transfected CHO cells in suspension as described (Linden et al., (1993) Mol. Pharm. 44:524-532). Supernatants (500 µL) were acetylated and acetylcyclic AMP was measured by automated radioimmunoassay (Hamilton, B.R. and Smith, D.O. (1991) J. Physiol. (Lond.) **432**, 327-341). Antagonist dissociation constants were estimated from pA_2 values as described by Schild (1957) Pharm. Rev. **9**, 242-246).

The ability of the HS-21a receptor stably expressed in CHO cells to couple to the cAMP regulatory system was measured using adenosine as an agonist. Adenosine (10 µM) produced a 30 % inhibition of the forskolin-stimulated increase in cAMP. In the absence of forskolin, adenosine had no effect on the cAMP levels. In non-transfected CHO cells, adenosine had no effect on cAMP levels when measured with or without forskolin treatment.

I-ABA produced only about half as much inhibition of forskolin-stimulated cyclic AMP accumulation in CHO cells as did NECA and other agonists (PIA and CPA). Furthermore, in the presence of I-ABA, the dose response curve of NECA to lower cyclic AMP was right-shifted. These data indicate that I-ABA is a partial agonist in this system. Dose-response curves of NECA-induced inhibition of forskolin-stimulated cAMP accumulation were also right shifted in the presence of competing xanthine antagonists (Figures 13A through 13F). Schild analyses were used to estimate the K_i from pA_2 values. The K_i values determined by competitive binding for various agonists and antagonists are compared with the K_A values in the functional cAMP assay in Table 1. The potency order profiles were nearly identical for the binding and functional assays, however, the K_a of agonists to lower cAMP were consistently higher (i.e. lower potency) than K_i values determined from competitive binding assays. Although the conditions of these assays differ, these data suggest that recombinant A3 receptors are not well coupled to inhibition of cyclic AMP accumulation in CHO cells.

EXAMPLE 13

Northern Blot Analysis

Human poly(A)$^+$ RNA from different tissue sources (Clontech) was fractionated on a 1% agarose-formaldehyde gel (Sambrook, J., Fritsch, E. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Press, Cold Spring Harbor, NY), transferred to Hybond-N membranes and hybridized in 5XSSPE, 5XDenhardt's, 0.5% SDS, 50 mg/mL sonicated salmon testis DNA, with 30% formamide (for A1, A2a, and A2b) or 50% formamide (for HS-21a) at 42°C. DNA probes corresponding to nucleotides 512-1614, 936-2168, and 321-1540 of accession numbers X68485(A1), X68486(A2a), and X68487(A2b) respectively, and a 1.7 kb SalI-BamHI fragment of HS-21a were labeled with a^{32}P-dCTP by the random priming method. Filters were washed under high stringency conditions in 0.1XSSC at 65°C.

Poly A$^+$ RNA from a number of human tissues was evaluated by Northern blot analyses to establish the distribution of tissue expression for the HS-21a transcript (Figure 14A). A 2 kb transcript was most abundantly expressed in lung and liver, with moderate amounts observed in brain and aorta. Low levels of expression were also observed in testis and heart. No expression was detected in spleen or kidney. The profile of lung=liver>>brain=aorta>testis>heart determined for HS-21a is considerably different from the tissue distribution of the other human adenosine receptor subtypes (Figure 14B). A human A1 transcript (2.9 kb) is expressed in brain, heart, kidney and lung with the most abundant expression observed in the brain. A second hybridizing band of 4.3 kb is also observed in lower amounts in the brain. In contrast to the results obtained for the A1 adenosine receptor, the A2a adenosine receptor transcript (2.8 kb) is equally expressed in brain, heart and kidney with slightly higher levels of expression detected in the lung. (Two hybridizing bands were observed when the full length A2a coding sequence was used as a probe and may be the result of cross-hybridization with the A1 transcript

(upper band)). In human brain, the expression of the A1 adenosine receptor subtype is most abundant in the cortex (25) and the expression of the A2a adenosine receptor subtype mRNA has been shown by in situ hybridization to be restricted to the caudate, putamen and nucleus accumbens (26). The human brain mRNA utilized in the Northern analysis was prepared from the brain stem, pons, cerebellum, telencephalon, diencephalon and mesencephalon regions of the brain and does not represent enriched transcripts from those regions of the brain in which the most abundant expression of A1 and A2a adenosine receptors has been indicated by radioligand binding or in situ hybridization studies. For the human A2b subtype, two hybridizing transcripts of 1.7kb and 2.1kb were observed in brain, heart and lung. The smaller 1.7kb transcript was more abundant. In contrast to the expression of the A1 and A2a adenosine receptor transcripts, no expression of A2b transcript was observed in the kidney. From the comparison of the distribution of human adenosine receptor transcripts, it can be concluded that the subtype transcripts are widely distributed but differ from each other in the abundance found in particular tissues.

EXAMPLE 14

SPECIFIC INHIBITION OF ADENOSINE INDUCED VASCULAR CONSTRICTION AND SPECIFIC INHIBITION OF ADENOSINE INDUCED MAST CELL DEGRANULATION

We have discovered that adenosine, adenosine metabolites and other A3 adenosine receptor agonists induce mast cell degranulation in an animal model and that this can be prevented by selective antagonists of the A3 receptor. The release of enzymes, bioactive amines and arachidonic acid metabolites following mast cell activation causes vasoconstriction, edema, leukocyte accumulation, and ultimately, tissue damage. Mast cell degranulation is a component of: myocardial reperfusion injuryu, hypersensitivity reactions (asthma, allergic rhinitis, and urticaria), ischemic bowel disease, autoimmune inflammationa, and atopic dermatitis. The invention consists of the use of any of a series of highly specific A3 adenosine receptor antagonists to treat or prevent

these diseases and pathologic effects that result from mast cell degranulation.

Adenosine is a potent vasodilator that has also been shown to cause vasoconstriction. The constrictor response has classically been attributed to A1 adenosine receptor stimulation or interactions with the renin-angiotensin system. A previously unreported vasoconstrictor action of adenosine in hamster cheek pouch arterioles is described here, and the specific blockade of this response by A3 adenosine receptor antagonists is demonstrated. Adenosine, inosine, cromolyn, compound 48/80, methylene blue, acetylcholine, and components for saline solutions used to bathe arterioles were obtained from Sigma. 8(p-sulphophenyl)theophylline was obtained from Research Biochemicals, Inc (Natick, MA).

Arterioles (luminal diameter approximately 60 μm) were dissected from male Golden hamster cheek pouches, transferred to a 37°C tissue chamber, and cannulated at both ends (see Duling et al., Am. J. Physiol. 241 (Heart Circ. Physiol. 10): H108-H116, 1981; Duling et al., Microcirculatory Technology, edited by C.H. Baker and W.G. Nastuk, Orlando Academic Press, 1986, p.265-280). Changes in luminal diameter in response to abluminal delivery of adenosine (10^{-8} M to 10^{-4} M) were measured using videotaped microscopic observation and video calipers with continuous output, to generate cumulative concentration-response curves. These curves were discovered to be biphasic: 10^{-6} M adenosine elicited an intense, transient constriction and higher concentrations induced dilator responses. Pretreatment (100 μM) with 8(p-sulfophenyl) theophylline, SPT, a nonspecific adenosine receptor antagonist, inhibited the dilator responses but did not alter the constriction. Without more, this result is consistent with the interpretation that the constrictor response is not mediated through an adenosine A1 or A2 receptor.

However, we made the critical observation that the constrictor response was assymetrical and focal in nature, such that it was initiated at discrete points and subsequently spread along the entire vessel, suggesting discrete sites of action of adenosine. Examination of

the abluminal surface of the vessel after staining with methylene blue revealed large numbers of mast cells closely associated with the vessel wall (figure 16). Following exposure to adenosine, mast cells were found to be degranulated, suggesting the involvement of mast cell granule contents in the constrictor response. This finding was consistent with reports that adenosine potentiates the release of granule contents from mast cells isolated from rat peritoneum (Lohse et al., N.-S. Arch. Pharmacol. 335:555-560, 1987; Marquardt et al., J. Immunol. 120:871-878, 1978), and that mast cell degranulation causes constriction in some vascular beds resulting in C5a-induced myocardial ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl. 578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem. 268:16887-16890, 1993). Accordingly, if a method were invented to specifically inhibit mast cell degranulation, a treatment or preventative method would be demonstrated for all of the above known and many yet to be defined disease states associated with adenosine induced mast cell degranulation.

We therefore tested the hypothesis that mast cells on the abluminal surface of the arteriole serve as foci of the constrictor response. We first tested this hypothesis by directly stimulating degranulation. Application of 2 μg/mL of compound 48/80, a mast cell secretagogue, induced a constriction similar to that observed with adenosine. Pretreatment of vessels with 10 μM cromolyn (sodium cromoglygate, a mast cell stabilizer) blocked the constrictor response induced by compound 48/80, see figures 18 and 19. Inhibition of degranulation was confirmed by observation of intact methylene blue-stained mast cells.

Adenosine A3 receptors have recently been found on mast cells. We tested the involvement of A3 receptors in the following manner: Treatment of the arterioles with the agonist IABA (30 μM, applied via pipette) resulted in vasoconstriction (see figure 17). Pretreatment with 10 μM BWA1433, characterized herein as an A3

specific adenosine receptor antagonist, resulted in complete abolition of the constrictor response and inhibition of mast cell degranulation (see figure 20). The A1 specific antagonist, 8PST (10 μM) had no effect on the IABA induced vasoconstriction, proving that the constrictor response is A3 receptor mediated and that this response can be blocked by contacting the A3 receptors exhibited on the mast cells with an A3 specific antagonist.

We therefore have shown that specific blockade of the A3 subtype of the adenosine receptor is effective to block the vasoconstrictive response induced through adenosine activation of this receptor subtype. We have also shown that use of a specific inhibitor of the A3 adenosine receptor subtype is effective to inhibit effects induced by adenosine mediated mast cell degranulation, and have therefore shown that disease states including but not limited to myocardial ischemia (Ito et al., Am. J. Physiol. 264 (Heart Circ. Physiol. 33):H1346-H1354, 1993), mast cell-dependent inflammation (Raud, J., Acta. Physiol. Scand. 135 (Suppl. 578):1-58, 1989), brain arteriole diameter constriction (Rosenblum, W. I., Brain Res. 49:75-82, 1973), and the release of allergic mediators (Ramkumar, et al., J. Biol. Chem. 268:16887-16890, 1993), are all amenable to prevention and treatment by contacting A3 receptor bearing mast cells with an amount of a selective A3 inhibitor effective to prevent mast cell degranulation.

EXAMPLE 15

EFFECT OF IABOPX ON MYOCARDIAL INFARCT SIZE IN ANESTHETIZED DOGS

The effect of3-(3-iodo-4-aminobenzyl)-8-oxyacetate-1-propyl-xanthine (IABOPX), a xanthine adenosine receptor antagonist with high affinity for canine A3 adenosine receptors (K_i=5.49 nM), on myocardial infarct size was compared to a vehicle-treated control group in barbital-anesthetized dogs subjected to 90 minutes of left anterior decending (LAD) coronary artery occlusion followed by 3 hours of reperfusion. Vehicle (5 μM NaOH in isotonic saline) or IABOPX (2 μM) were infused at a rate of 1 ml/min directly into the LAD coronary

artery distal to the occlusion site beginning 10 minutes before occlusion and were continued throughout the entire ischemic period. The myocardial region at risk and infarct size were determined by the triphenyltetrazolium histochemical technique and regional myocardial blood flow by radioactive microspheres. Coronary sinus LDH activity and histamine concentrations were measured at various times throughout the experiments and myeloperoxidase activity (MPO) was determined at the conclusion of the experiments as an index of neutrophil infiltration into the ischemic-reperfused mycocardium. Ten dogs were included in this preliminary study, 4 in the vehicle-treated group and 6 in the IABOPX-treated group.

In all animals, arrhythmias were encountered during occlusion and reperfusion and 3 of 4 dogs progressed to ventricular fibrillation requiring cardioversion in the vehicle-treated group and 3 of 6 in the IABOPX-treated group (Table 1). Cardioversion was unsuccessful in 2 experiments in which the drug was administered thus data analysis was performed in 8 animals, 4 in each group. Hemodynamics (Table 2) and regional myocardial blood flow in the non-ischemic left circumflex coronary artery region (Table 3) were not different between groups at baseline or during occlusion although during reperfusion dP/dt was significantly improved and LAD coronary artery blood flow was significantly decreased in IABOPX-treated dogs. In contrast, in the ischemic-reperfused region collateral blood flow during the occlusion period, the major determinant of ultimate infarct size, was slightly greater in drug-treated dogs particularly in the subepicardial region (Table 4, Figure 1). The area at risk of infarction was not different between groups: vehicle, 33.3 $\pm$ 5.0%; IABOPX, 26.8 $\pm$ 2.4%, however, myocardial infarct size expressed as a percent of the area at risk (vehicle, 48.1 $\pm$ 2.5; 20.4 $\pm$ 11.2) was significantly smaller in the IABOPX-treated group (Figure 2) and this beneficial effect of IABOPX did not appear to be the result of enhanced collateral blood flow (Figure 3). In addition, coronary sinus LDH activity (Figure 4) and histamine concentrations (Figure 5) were strikingly reduced in the

IABOPX-treated group during reperfusion. Finally, MPO activity was not different between the two groups although there was a tendency for reduced activity in drug-treated animals in infarcted tissue (Figure 6). Taken together, these preliminary results suggest that IABOPX effectively reduces myocardial infarct size in anesthetized dogs by a direct cardioprotective action most likely by inhibiting mast cell degranulation secondary to blockade of A3 adenosine receptors.

TABLE 1

Mortailty and Incidence of Ventricular Fibrillation

	MORTALITY	FIBRILLATIONS		
		Occ	Rep	Total
VEHICLE	0/4	3/4	1/4	3/4
IABOPX	2/6	1/6	2/6	3/6

TABLE 2
HEMODYNAMICS

	HR (beat/min)	MAP (mm Hg)	LVd/dt (mm Hg/sec)	CBF (ml/min)
BASELINE				
VEHICLE	134 ± 10	103 ± 6	488 ± 16	35 ± 4
IABOPX	124 ± 11	96 ± 7	544 ± 62	33 ± 5
OCCLUSION (75 min)				
VEHICLE	130 ± 11	113 ± 7	417 ± 14	-----
IABOPX	121 ± 9	99 ± 5	464 ± 53	-----
REPERFUSION (5 min)				
VEHICLE	129 ± 8	96 ± 6	384 ± 45	101 ± 14†
IABOPX	128 ± 8	96 ± 5	448 ± 49†	99 ± 8†
REPERFUSION (3 hr)				
VEHICLE	114 ± 9†	99 ± 5	346 ± 19†	45 ± 4
IABOPX	116 ± 13	108 ± 4†	494 ± 54*	31 ± 2*

All values are the mean ± SEM (Vehicle n=4; IABOPX n=4)
HR=heart rate; MAP=mean arterial blood pressure;
LVdP/dt=left ventricular dP/dt; CBF=LAD coronary blood flow
*$p<0.05$ vs. vehicle group (ANOVA and Fisher's LSD)
†$p<0.05$ vs. baseline value within groups (ANOVA with repeated measures and Dunnet's t-test)

TABLE 3
REGIONAL MYOCARDIAL BLOOD FLOW
IN THE NORMAL REGION

	MYOCARDIAL BLOOD FLOW (ml/min/g)		
	EPI	MID	ENDO
BASELINE			
VEHICLE	1.04 ± 0.04	0.96 ± 0.02	1.01 ± 0.04
IABOPX	0.96 ± 0.08	0.91 ± 0.04	0.94 ± 0.04

OCCLUSION (75 min)			
VEHICLE	0.98 ± 0.04	0.92 ± 0.09	0.97 ± 0.03
IABOPX	1.05 ± 0.09	0.93 ± 0.05	1.30 ± 0.20
REPERFUSION (3 hr)			
VEHICLE	1.04± 0.03	0.96 ± 0.04	0.95 ± 0.06
IABOPX	1.16 ± 0.15	1.15 ± 0.18	1.17 ± 0.18

All values are the mean ± S.E.M. (Vehicle n=4; IABOPX n=4)
EPI=epicardium; MID=midmyocardium; ENDO=endocardium

TABLE 4
REGIONAL MYOCARDIAL BLOOD FLOW
IN THE ISCHEMIC REGION

	MYOCARDIAL BLOOD FLOW (ml/min/g)		
	EPI	MID	ENDO
BASELINE			
VEHICLE	0.97 ± 0.04	0.84 ± 0.04	0.85 ± 0.02
IABOPX	0.95 ± 0.13	0.76 ± 0.06	0.71 ± 0.05*
OCCLUSION (75 min)			
VEHICLE	0.20 ± 0.04†	0.11 ± 0.04†	0.08 ± 0.04†
IABOPX	0.47 ± 0.10*†	0.22 ± 0.06†	0.13 ± 0.04†
REPERFUSION (3 hr)			
VEHICLE	0.95 ± 0.14	1.29 ± 0.17†	2.00 ± 0.28†
IABOPX	1.11 ± 0.14	0.86 ± 0.07	1.43 ± 0.21†

All values are the mean ± S.E.M. (Vehicle n=4; IABOPX n=4)
EPI=epicardium; MID=midmyocardium; ENDO=endocardium
*$p<0.05$ vs. vehicle group (ANOVA and Fisher's LSD)
†$p<0.05$ vs. baseline value within groups (ANOVA with repeated measures and Dunnet's t-test)

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention

encompasses all of the usual variations, adaptations, modifications, as come within the scope of the following claims and its equivalents.

WHAT IS CLAIMED IS:

1. A method for achieving blockade of the vasoconstrictive response induced through adenosine activation of the A3 adenosine receptor subtype which comprises contacting cells bearing said receptor with an amount of an adenosine A3 receptor subtype specific inhibitor effective to block activation of said receptor by adenosine.

2. A method for treating or preventing myocardial ischemia, inflammation, brain arteriole diameter constriction, and the release of allergic mediators, which comprises using a specific inhibitor of the A3 adenosine receptor subtype to inhibit effects induced by adenosine mediated mast cell degranulation by contacting A3 receptor bearing mast cells with an amount of a selective A3 inhibitor effective to prevent mast cell degranulation.

3. A method for preventing or treating asthma, bronchoconstriction, allergic potentiation, inflamation or reperfusion injury in a human which comprises administering an amount of a xanthine or a xanthine derivative having an acidic aryl at the 8 position and a substituted or unsubstituted aryl, alkyl or alkenyl substituent at the 3 position effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.

4. A method for preventing mast cell degranulation in a human which comprises administering an amount of a xanthine or a xanthine derivative having an acidic aryl at the 8 position and a substituted or unsubstituted aryl, alkyl or alkenyl substituent at the 3 position effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.

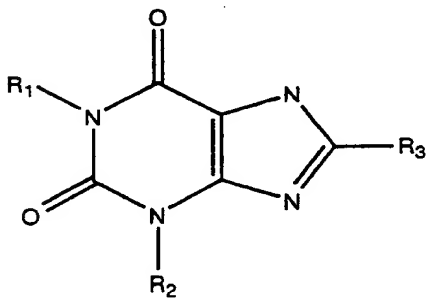
5. A method for achieving blockade of vascular constriction induced through activation of the A3 subtype of the

adenosine receptor in a primate which comprises contacting said receptor in the vasculature with an amount of a xanthine or xanthine derivative effective to reduce vasoconstriction in the vasculature without any substantial effect (binding or blockade) of the A1 or A2 subtypes of the adenosine receptor.

6. The method of claim 5 which comprises the treatment or prevention of disease states mediated through activation of the A3 subtype of the adenosine receptor on mast cells by prevention of mast cell degranulation through blockade of the A3 subtype of the adenosine receptor by contacting mast cells with an inhibitory effective amount of a xanthine or xanthine derivative specific for the A3 receptor subtype.

7. The method of claim 6 wherein the disease state associated with A3 adenosine receptor activation and mast cell degranulation includes asthma, myocardial reperfusion injury, allergic reactions including but not limited to rhinitis, poison ivy induced responses, urticaria, scleroderma, arthritis, and inflammatory bowel diseases.

8. The method of any one of Claims 3-7 wherein said xanthine has the formula:

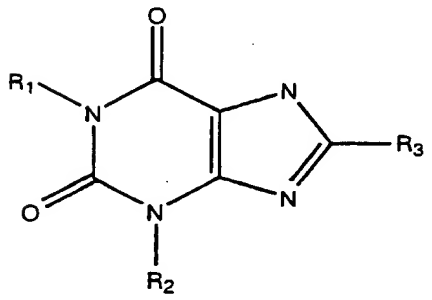


wherein R_1, R_2, and R_3, independently, are as defined below:

R_1	R_2	R_3
alkyl	aryl	acidic aryl
alkenyl	substituted aryl	substituted acidic aryl
cycloalkyl	hetero-aryl	
	substituted hetero-aryl	

wherein:
alkyl,alkenyl,cycloalkyl is substituted or unsubstituted
aryl is benzyl, phenyl;
substituted aryl is an aryl substituted with an alkyl, amino or halogen; and
acidic aryl is an aryl substituted with a carboxylate, oxyacetate, acrylate, sulphonate, phosphonate, or tetrazol.

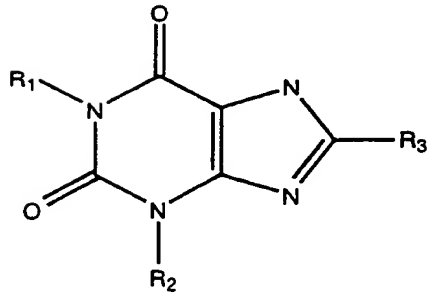
9. The method of claim 8 wherein said xanthine is:



wherein R_1, R_2, and R_3, independently, are as defined below:

R_1	R_2	R_3
lower alkyl	benzyl halogenated benzyl amino-benzyl halogenated amino-benzyl.	benzyl-acid

10. The method of Claim 9 wherein said xanthine is:



wherein R_1, R_2, and R_3, independently, are as defined below:

R_1	R_2	R_3
$-C_3H_7$	$-C_3H_7$	$-CH_2-C_6H_4-O$-acid
$-CH_3$	-benzyl	$-CH_2-COO^-$
$-C_2H_5$	-halogenated benzyl	-indole
	-aminobenzyl	
	-halogenated aminobenzyl	

wherein said acid is -indole, -carboxylate, sulphonate, phosphonate.

11. The method of any one of claims 3-5 wherein the xanthine is selected from the group consisting of IABOPX, BW-A1433, BW-934, and BW-A215.

12. A method for preventing or treating asthma, bronchoconstriction, allergic potentiation, inflamation or reperfusion injury in a human which comprises administering an amount of a xanthine or a xanthine derivative having an affinity for the A3 subtype of the human adenosine receptor which is at least one order of magnitude greater than the affinity for either the A1 A2a or A2b subtypes of the human adenosine receptor effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.

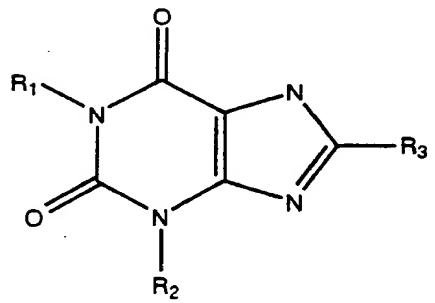
13. A method for preventing mast cell degranulation in a human which comprises administering an amount of a xanthine or a xanthine derivative having an affinity for the A3 subtype of the human adenosine receptor which is at least one order of magnitude greater than the affinity for either the A1 or A2 subtypes of the human adenosine receptor effective to antagonize activation of the adenosine receptor of the A3 subtype by adenosine.

14. A method for achieving blockade of vascular constriction induced through activation of the A3 subtype of the adenosine receptor in a primate which comprises contacting said receptor in the vasculature with an amount of a xanthine or xanthine derivative having an affinity for the A3 subtype of the human adenosine receptor which is at least one order of magnitude greater than the affinity for either the A1 A2a or A2b subtypes of the human adenosine receptor effective to reduce vasoconstriction in the vasculature without any substantial effect (binding or blockade) of the A1 A2a or A2b subtypes of the adenosine receptor.

15. The method of claim 14 wherein said vasculature is in the lung.

16. A method for achieving blockade of the A3 subtype of the adenosine receptor in a primate which comprises contacting said receptor with an amount of a xanthine or xanthine derivative effective to inhibit activation of said receptor by adenosine, without any substantial effect (binding or blockade) of other subtypes of the adenosine receptor.

17. The method of Claim 16 wherein said xanthine has the formula:

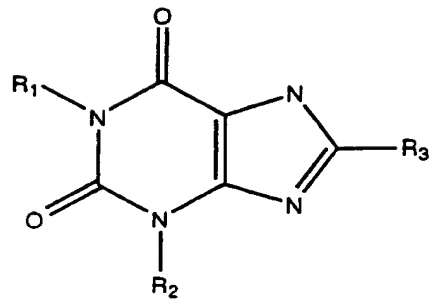


wherein R_1, R_2, and R_3, independently, are as defined below:

R_1	R_2	R_3
alkyl	aryl	acidic aryl
alkenyl	substituted aryl	substituted acidic aryl
cycloalkyl	hetero-aryl	
	substituted hetero-aryl	

wherein:
alkyl,alkenyl,cycloalkyl is substituted or unsubstituted
aryl is benzyl, phenyl;
substituted aryl is an aryl substituted with an alkyl, amino or halogen; and
acidic aryl is an aryl substituted with a carboxylate, oxyacetate, acrylate, sulphonate, phosphonate, or tetrazol.

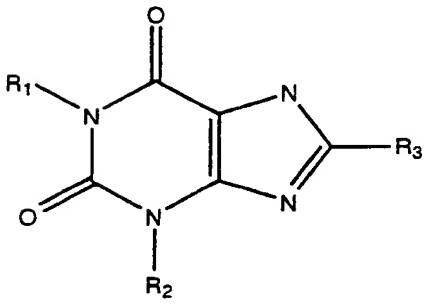
18. The method of claim 16 wherein said xanthine is:



wherein R1, R2, and R3, independently, are as defined below:

R1	R2	R3
lower alkyl	benzyl halogenated benzyl amino-benzyl halogenated amino-benzyl.	benzyl-acid

19. The method of Claim 16 wherein said xanthine is:



wherein R_1, R_2, and R_3, independently, are as defined below:

R_1	R_2	R_3
$-C_3H_7$	$-C_3H_7$	$-CH_2-C_6H_4-O$-acid
$-CH_3$	-benzyl	$-CH_2-COO^-$
$-C_2H_5$	-halogenated benzyl	-indole
	-aminobenzyl	
	-halogenated aminobenzyl	

wherein said acid is -indole, -carboxylate, sulphonate, phosphonate.

20. The method of Claim 16 wherein the xanthine is selected from the group consisting of IABOPX, BW-A1433, BW-934, and BW-A215.

21. The method of Claim 16 wherein the xanthine has a pKi for the A3 sutype of 7 or greater, and a pKi for other adenosine receptor subtypes of 6 or less.

22. A method for achieving blockade of the A3 subtype of the adenosine receptor in a primate which comprises contacting said receptor with an amount of an A3 adenosine receptor antagonist having a pKi for the A3 sutype of 7 or greater, and a pKi for other adenosine receptor subtypes of 6 or less.

23. A method for treating an autoimmune disease selected from the group consisting of Addison's disease (adrenal), autoimmune hemolytic anemia (red cells), Crohn's disease (gut), Goodpasture's syndrome (kidney and lungs), Grave's disease (thyroid), Hashimoto's thyroiditis (thyroid), idiopathic thrombocytopinic purpura (platelets). Insulin-dependent diabetes militus (pancreatic beta cells), multiple sclerosis (brain and spinal cord), myasthenia gravis

(nerve/muscle synapses), Pemphigus vulgaris (skin), pernicious anemia (gastric parietal cells), poststreptococcal glomerulonephritis (kidney), psoriasis (skin), rheumatoid arthritis (connective tissue), scleroderma (heart, lung, gut, kidney), Sjogren's syndrome (liver, kidney, brain, thyroid, salivary gland), spontaneous infertility (sperm), and systemic lupus erythematosus (DNA, platelets, other tissues), which comprises administration of selective A3 adenosine receptor antagonists effective to inhibit mast cell degranulation.

10 20
Met Pro Pro Ser Ile Ser Ala Phe Gln Ala Ala Tyr Ile Gly Ile Glu Val Leu Ile Ala
30 40
Leu Val Ser Val Pro Gly Asn Val Leu Val Ile Trp Ala Val Lys Val Asn Gln Ala Leu
50 60
Arg Asp Ala Thr Phe Cys Phe Ile Val Ser Leu Ala Val Ala Asp Val Ala Val Gly Ala
70 80
Leu Val Ile Pro Leu Ala Ile Leu Ile Asn Ile Gly Pro Gln Thr Tyr Phe His Thr Cys
90 100
Leu Met Val Ala Cys Pro Val Leu Ile Leu Thr Gln Ser Ser Ile Leu Ala Leu Leu Ala
110 120
Ile Ala Val Asp Arg Tyr Leu Arg Val Lys Ile Pro Leu Arg Tyr Lys Met Val Val Thr
130 140
Pro Arg Arg Ala Ala Val Ala Ile Ala Gly Cys Trp Ile Leu Ser Phe Val Val Gly Leu
150 160
Thr Pro Met Phe Gly Trp Asn Asn Leu Ser Ala Val Glu Arg Ala Trp Ala Ala Asn Gly
170 180
Ser Met Gly Glu Pro Val Ile Lys Cys Glu Phe Glu Lys Val Ile Ser Met Glu Tyr Met
190 200
Val Tyr Phe Asn Phe Phe Val Trp Val Leu Pro Pro Leu Leu Leu Met Val Leu Ile Tyr
210 220
Leu Glu Val Phe Tyr Leu Ile Arg Lys Gln Leu Asn Lys Lys Val Ser Ala Ser Ser Gly
230 240
Asp Pro Gln Lys Tyr Tyr Gly Lys Glu Leu Lys Ile Ala Lys Ser Leu Ala Leu Ile Leu
250 260
Phe Leu Phe Ala Leu Ser Trp Leu Pro Leu His Ile Leu Asn Cys Ile Thr Leu Phe Cys
270 280
Pro Ser Cys His Lys Pro Ser Ile Leu Thr Tyr Ile Ala Ile Phe Leu Thr His Gly Asn
290 300
Ser Ala Met Asn Pro Ile Val Tyr Ala Phe Arg Ile Gln Lys Phe Arg Val Thr Phe Leu
310 320
Lys Ile Trp Asn Asp His Phe Arg Cys Gln Pro Ala Pro Pro Ile Asp Glu Asp Leu Pro
326
Glu Glu Arg Pro Asp Asp

FIG. 1

```
         10                    30                    50
atgccgccct ccatctcagc tttccaggcc gcctacatcg gcatcgaggt gctcatcgcc
         70                    90                   110
ctggtctctg tgcccgggaa cgtgctggtg atctgggcgg tgaaggtgaa ccaggcgctg
        130                   150                   170
cgggatgcca ccttctgctt catcgtgtcg ctggcggtgg ctgatgtggc cgtgggtgcc
        190                   210                   230
ctggtcatcc ccctcgccat cctcatcaac attgggccac agacctactt ccacacctgc
        250                   270                   290
ctcatggttg cctgtccggt cctcatcctc acccagagct ccatcctggc cctgctggca
        310                   330                   350
attgctgtgg accgctacct ccgggtcaag atccctctcc ggtacaagat ggtggtgacc
        370                   390                   410
ccccggaggg cggcggtggc catagccggc tgctggatcc tctccttcgt ggtgggactg
        430                   450                   470
acccctatgt ttggctggaa caatctgagt gcggtggagc gggcctgggc agccaacggc
        490                   510                   530
agcatggggg agcccgtgat caagtgcgag ttcgagaagg tcatcagcat ggagtacatg
        550                   570                   590
gtctacttca acttctttgt gtgggtgctg cccccgcttc tcctcatggt cctcatctac
        610                   630                   650
ctggaggtct tctacctaat ccgcaagcag ctcaacaaga aggtgtcggc ctcctccggc
        670                   690                   710
```

FIG. 2A

```
gacccgcaga agtactatgg gaaggagctg aagatcgcca agtcgctggc cctcatcctc
      730        750        770        790        810        830
ttcctctttg ccctcagctg gctgcctttg cacatcctca actgcatcac cctcttctgc
      790        810        830        850        870        890
ccgtcctgcc acaagcccag catccttacc tacattgcca tcttcctcac gcacggcaac
      850        870        890        910        930        950
tcggccatga accccattgt ctatgccttc cgcatccaga agttccgcgt caccttcctt
      910        930        950        970
aagatttgga atgaccattt ccgctgccag cctgcacctc ccattgacga ggatctccca
      970
gaagagaggc ctgatgacta g
```

FIG. 2B

10 20
Met Pro Ile Met Gly Ser Ser Val Tyr Ile Thr Val Glu Leu Ala Ile Ala Val Leu Ala
30 40
Ile Leu Gly Asn Val Leu Val Cys Trp Ala Val Trp Leu Asn Ser Asn Leu Gln Asn Val
50 60
Thr Asn Tyr Phe Val Val Ser Leu Ala Ala Ala Asp Ile Ala Val Gly Val Leu Ala Ile
70 80
Pro Phe Ala Ile Thr Ile Ser Thr Gly Phe Cys Ala Ala Cys His Gly Cys Leu Phe Ile
90 100
Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu Leu Ala Ile Ala Ile
110 120
Asp Arg Tyr Ile Ala Ile Arg Ile Pro Leu Arg Tyr Asn Gly Leu Val Thr Gly Thr Arg
130 140
Ala Lys Gly Ile Ile Ala Ile Cys Trp Val Leu Ser Phe Ala Ile Gly Leu Thr Pro Met
150 160
Leu Gly Trp Asn Asn Cys Gly Gln Pro Lys Glu Gly Lys Asn His Ser Gln Gly Cys Gly
170 180
Glu Gly Gln Val Ala Cys Leu Phe Glu Asp Val Val Pro Met Asn Tyr Met Val Tyr Phe
190 200
Asn Phe Phe Ala Cys Val Leu Val Pro Leu Leu Leu Met Leu Gly Val Tyr Leu Arg Ile
210 220
Phe Leu Ala Ala Arg Arg Gln Leu Lys Gln Met Glu Ser Gln Pro Leu Pro Gly Glu Arg
230 240
Ala Arg Ser Thr Leu Gln Lys Glu Val His Ala Ala Lys Ser Leu Ala Ile Ile Val Gly
250 260
Leu Phe Ala Leu Cys Trp Leu Pro Leu His Ile Ile Asn Cys Phe Thr Phe Phe Cys Pro
270 280
Asp Cys Ser His Ala Pro Leu Trp Leu Met Tyr Leu Ala Ile Val Leu Ser His Thr Asn
290 300
Ser Val Val Asn Pro Phe Ile Tyr Ala Tyr Arg Ile Arg Glu Phe Arg Gln Thr Phe Arg
310 320
Lys Ile Ile Arg Ser His Val Leu Arg Gln Gln Glu Pro Phe Lys Ala Ala Gly Thr Ser
330 340
Ala Arg Val Leu Ala Ala His Gly Ser Asp Gly Glu Gln Val Ser Leu Arg Leu Asn Gly
350 360
His Pro Pro Gly Val Trp Ala Asn Gly Ser Ala Pro His Pro Glu Arg Arg Pro Asn Gly
370 380
Tyr Ala Leu Gly Leu Val Ser Gly Gly Ser Ala Gln Glu Ser Gln Gly Asn Thr Gly Leu
390 400
Pro Asp Val Glu Leu Leu Ser His Glu Leu Lys Gly Val Cys Pro Glu Pro Pro Gly Leu
410
Asp Asp Pro Leu Ala Gln Asp Gly Ala Gly Val Ser

FIG. 3

```
         10                       30                       50
atgcccatca tgggctcctc ggtgtacatc acggtggagc tggccattgc tgtgctggcc
         70                       90                      110
atcctgggca atgtgctggt gtgctgggcc gtgtggctca acagcaacct gcagaacgtc
        130                      150                      170
accaactact ttgtggtgtc actggcggcg gccgacatcg cagtgggtgt gctcgccatc
        190                      210                      230
ccctttgcca tcaccatcag caccgggttc tgcgctgcct gccacggctg cctcttcatt
        250                      270                      290
gcctgcttcg tcctggtcct cacgcagagc tccatcttca gtctcctggc catcgccatt
        310                      330                      350
gaccgctaca ttgccatccg catcccgctc cggtacaatg gcttggtgac cggcacgagg
        370                      390                      410
gctaagggca tcattgccat ctgctgggtg ctgtcgtttg ccatcggcct gactcccatg
        430                      450                      470
ctaggttgga acaactgcgg tcagccaaag gagggcaaga accactccca gggctgcggg
        490                      510                      530
gagggccaag tggcctgtct ctttgaggat gtggtcccca tgaactacat ggtgtacttc
        550                      570                      590
aacttctttg cctgtgtgct ggtgcccctg ctgctcatgc tgggtgtcta tttgcggatc
        610                      630                      650
ttcctggcgg cgcgacgaca gctgaagcag atggagagcc agcctctgcc gggggagcgg
        670                      690                      710
```

FIG. 4A

```
gcacggtcca cactgcagaa ggaggtccat gctgccaagt cactggccat cattgtgggg
      730                 750                 770
ctctttgccc tctgctggct gcccctacac atcatcaact gcttcacttt cttctgcccc
      790                 810                 830
gactgcagcc acgcccctct ctggctcatg tacctggcca tcgtcctctc ccacaccaat
      850                 870                 890
tcggttgtga atcccttcat ctacgcctac cgtatccgcg agttccgcca gaccttccgc
      910                 930                 950
aagatcattc gcagccacgt cctgaggcag caagaacctt tcaaggcagc tggcaccagt
      970                 990                1010
gcccgggtct tggcagctca tggcagtgac ggagagcagg tcagcctccg tctcaacggc
     1030                1050                1070
cacccgccag gagtgtgggc caacggcagt gctccccacc ctgagcggag gcccaatggc
     1090                1110                1130
tatgccctgg ggctggtgag tggagggagt gcccaagagt cccaggggaa cacgggcctc
     1150                1170                1190
ccagacgtgg agctccttag ccatgagctc aagggagtgt gcccagagcc ccctggccta
     1210                1230
gatgacccccc tggcccagga tggagcagga gtgtcctga
```

FIG. 4B

10 20
Met Leu Leu Glu Thr Gln Asp Ala Leu Tyr Val Ala Leu Glu Leu Val Ile Ala Ala Leu

30 40
Ser Val Ala Gly Asn Val Leu Val Cys Ala Ala Val Gly Thr Ala Asn Thr Leu Gln Thr

50 60
Pro Thr Asn Tyr Phe Leu Val Ser Leu Ala Ala Ala Asp Val Ala Val Gly Leu Phe Ala

70 80
Ile Pro Phe Ala Ile Thr Ile Ser Leu Gly Phe Cys Thr Asp Phe Tyr Gly Cys Leu Phe

90 100
Leu Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu Leu Ala Val Ala

110 120
Val Asp Arg Tyr Leu Ala Ile Cys Val Pro Leu Arg Tyr Lys Ser Leu Val Thr Gly Thr

130 140
Arg Ala Arg Gly Val Ile Ala Val Leu Trp Val Leu Ala Phe Gly Ile Gly Leu Thr Pro

150 160
Phe Leu Gly Trp Asn Ser Lys Asp Ser Ala Thr Asn Asn Cys Thr Glu Pro Trp Asp Gly

170 180
Thr Thr Asn Glu Ser Cys Cys Leu Val Lys Cys Leu Phe Glu Asn Val Val Pro Met Ser

190 200
Tyr Met Val Tyr Phe Asn Phe Phe Gly Cys Val Leu Pro Pro Leu Leu Ile Met Leu Val

210 220
Ile Tyr Ile Lys Ile Phe Leu Val Ala Cys Arg Gln Leu Gln Arg The Glu Leu Met Asp

230 240
His Ser Arg Thr Thr Leu Gln Arg Glu Ile His Ala Ala Lys Ser Leu Ala Met Ile Val

250 260
Gly Ile Phe Ala Leu Cys Trp Leu Pro Val His Ala Val Asn Cys Val Thr Leu Phe Gln

270 280
Pro Ala Gln Gly Lys Asn Lys Pro Lys Trp Ala Met Asn Met Ala Ile Leu Leu Ser His

290 300
Ala Asn Ser Val Val Asn Pro Ile Val Tyr Ala Tyr Arg Asn Arg Asp Phe Arg Tyr Thr

310 320
Phe His Lys Ile Ile Ser Arg Tyr Leu Leu Cys Gln Ala Asp Val Lys Ser Gly Asn Gly

330
Gln Ala Gly Val Gln Pro Ala Leu Gly Val Gly Leu

FIG. 5

```
        10                            30                            50
atgctgctgg agacacagga cgcgctgtac gtggcgctgg agctggtcat cgccgcgctt
        70                            90                           110
tcggtggcgg gcaacgtgct ggtgtgcgcc gcggtgggca cggcgaacac tctgcagacg
       130                           150                           170
cccaccaact acttcctggt gtccctggct gcggccgacg tggccgtggg gctcttcgcc
       190                           210                           230
atcccctttg ccatcaccat cagcctgggc ttctgcactg acttctacgg ctgcctcttc
       250                           270                           290
ctcgcctgct tcgtgctggt gctcacgcag agctccatct tcagccttct ggccgtggca
       310                           330                           350
gtcgacagat acctggccat ctgtgtcccg ctcaggtata aagtttggt cacggggacc
       370                           390                           410
cgagcaagag ggtcattgc tgtcctctgg gtccttgcct ttggcatcgg attgactcca
       430                           450                           470
ttcctggggt ggaacagtaa agacagtgcc accaacaact gcacagaacc ctgggatgga
       490                           510                           530
accacgaatg aaagctgctg ccttgtgaag tgtctctttg agaatgtggt ccccatgagc
       550                           570                           590
tacatggtat atttcaattt ctttgggtgt gttctgcccc cactgcttat aatgctggtg
       610                           630                           650
atctacatta agatcttcct ggtggcctgc aggcagcttc agcgcactga gctgatggac
       670                           690                           710
```

FIG. 6A

```
cactcgagga ccaccctcca gcgggagatc catgcagcca agtcactggc catgattgtg
      730                 750                 770
gggatttttg ccctgtgctg gttacctgtg catgctgtta actgtgtcac tctttttccag
      790                 810                 830
ccagctcagg gtaaaaataa gcccaagtgg gcaatgaata tggccattct tctgtcacat
      850                 870                 890
gccaattcag ttgtcaatcc cattgtctat gcttaccgga accgagactt ccgctacact
      910                 930                 950
ttcacaaaaa ttatctccag gtatcttctc tgccaagcag atgtcaagag tgggaatggt
      970                 990
caggctgggg tacagccctgc tctcggtgtg ggcctatga
```

FIG. 6B

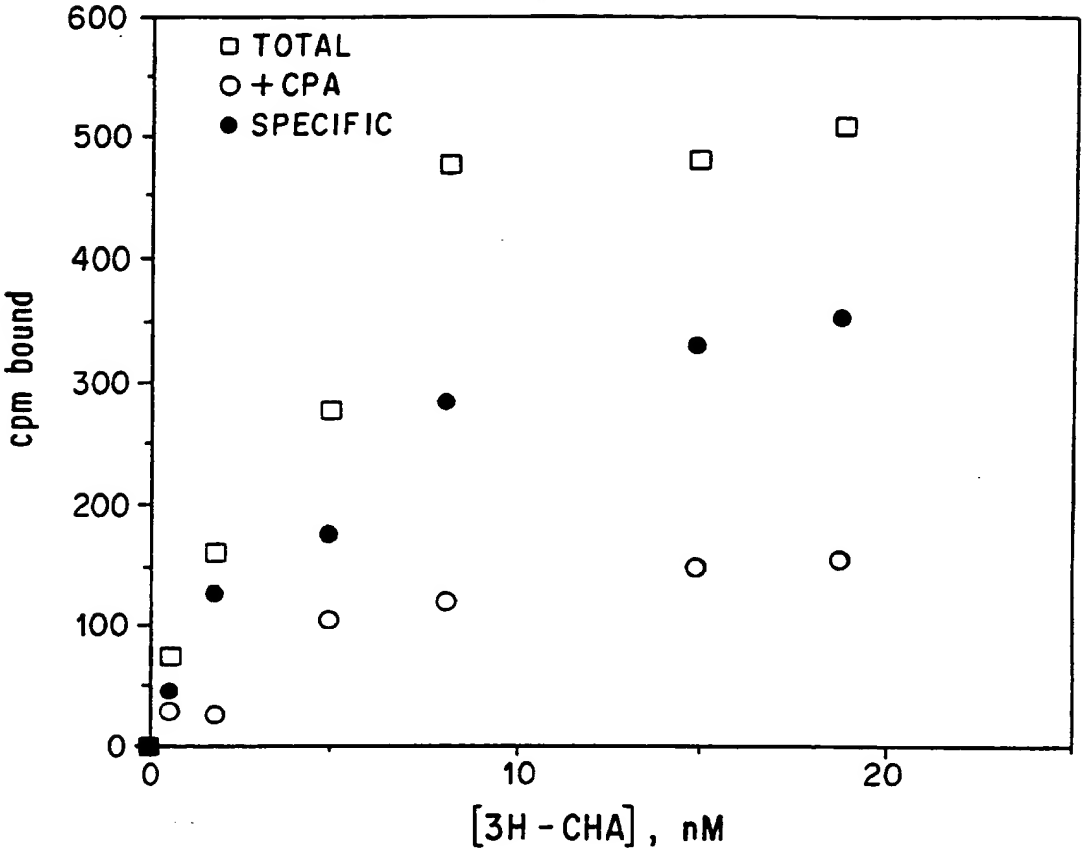

600
500
400
300
200
100
0
cpm bound
TOTAL
+CPA
SPECIFIC
0
10
20
[3H-CHA], nM

FIG. 7

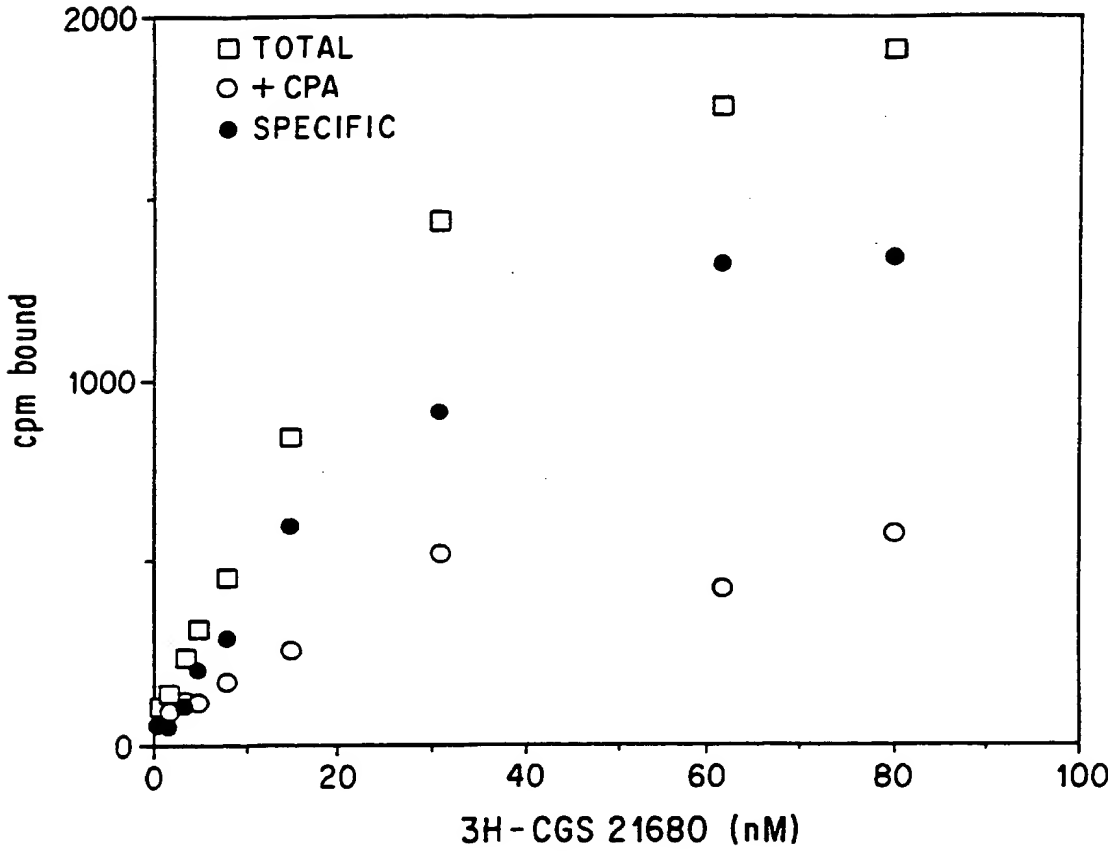

2000
1000
0
cpm bound
TOTAL
+ CPA
SPECIFIC
0
20
40
60
80
100
3H-CGS 21680 (nM)

FIG. 8

10 20
Met Pro Asn Asn Ser Thr Ala Leu Ser Leu Ala Asn Val Thr Tyr Ile Thr Met Glu Ile
30 40
Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu Val Ile Cys Val Val Lys Leu Asn
50 60
Pro Ser Leu Gln Thr Thr Thr Phe Tyr Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala
70 80
Val Gly Val Leu Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile His Phe
90 100
Tyr Ser Cys Leu Phe Met Thr Cys Leu Leu Leu Ile Phe Thr His Ala Ser Ile Met Ser
110 120
Leu Leu Ala Ile Ala Val Asp Arg Tyr Leu Arg Val Lys Leu Thr Val Arg Tyr Lys Arg
130 140
Val Thr Thr His Arg Arg Ile Trp Leu Ala Leu Gly Leu Cys Trp Leu Val Ser Phe Leu
150 160
Val Gly Leu Thr Pro Met Phe Gly Trp Asn Met Lys Leu Thr Ser Glu Tyr His Arg Asn
170 180
Val Thr Phe Leu Ser Cys Gln Phe Val Ser Val Met Arg Met Asp Tyr Met Val Tyr Phe
190 200
Ser Phe Leu Thr Trp Ile Phe Ile Pro Leu Val Val Met Cys Ala Ile Tyr Leu Asp Ile
210 220
Phe Tyr Ile Ile Arg Asn Lys Leu Ser Leu Asn Leu Ser Asn Ser Lys Glu Thr Gly Ala
230 240
Phe Tyr Gly Arg Glu Phe Lys Thr Ala Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala
250 260
Leu Ser Trp Leu Pro Leu Ser Ile Ile Asn Cys Ile Ile Tyr Phe Asn Gly Glu Val Pro
270 280
Gln Leu Val Leu Tyr Met Gly Ile Leu Leu Ser His Ala Asn Ser Met Met Asn Pro Ile
290 300
Val Tyr Ala Tyr Lys Ile Lys Lys Phe Lys Glu Thr Tyr Leu Leu Ile Leu Lys Ala Cys
310
Val Val Cys His Pro Ser Asp Ser Leu Asp Thr Ser Ile Glu Lys Asn Ser Glu

FIG. 9

```
10                  30                  50
atgcccaaca acagcactgc tctgtcattg gccaatgtta cctacatcac catggaaatt
    70         90         110         130
ttcattggac tctgcgccat agtgggcaac gtgctggtca tctgcgtggt caagctgaac
   130         150         170         190
cccagcctgc agaccaccac cttctatttc attgtctctc tagcccttggc tgacattgct
   190         210         230         250
gttgggggtgc tggtcatgcc tttggccatt gttgtcagcc tgggcatcac aatccacttc
   250         270         290         310
tacagctgcc ttttttatgac ttgcctactg cttatctttta cccacgcctc catcatgtcc
   310         330         350         370
ttgctggcca tcgctgtgga ccgatacttg cgggtcaagc ttaccgtcag atacaagagg
   370         390         410         430
gtcaccactc acagaagaat atggctggcc ctgggccttt gctggctggt gtcattcctg
   430         450         470         490
gtgggattga ccccatgtt tggctggaac atgaaactga cctcagagta ccacagaaat
   490         510         530         550
gtcaccttcc tttcatgcca atttgtttcc gtcatgagaa tggactacat ggtatacttc
   550         570         590         610
agcttcctca cctggatttt catccccctg gttgtcatgt gcgccatcta tcttgacatc
   610         630         650         670
ttttacatca ttcggaacaa actcagtctg aacttatcta actcccaaga gacaggtgca
   670         690         710
```

FIG. 10A

```
tttatggac  gggagttcaa  gacggctaag  tccttgtttc  tggttctttt  cttgtttgct
    730         790         750         770         770
ctgtcatggc  tgccttttatc  tatcatcaac  tgcatcatct  actttaatgg  tgaggtacca
    790         790         810         830         830
cagcttgtgc  tgtacatggg  catcctgctg  tcccatgcca  actccatgat  gaaccctatc
    850         850         870         890         890
gtctatgcct  ataaaataaa  gaagttcaag  gaaacctacc  ttttgatcct  caaagcctgt
    910         910         930         950         950
gtggtcctgcc  atcctctga  ttctttggac  acaagcattg  agaagaattc  tgagtag
```

FIG. 10B

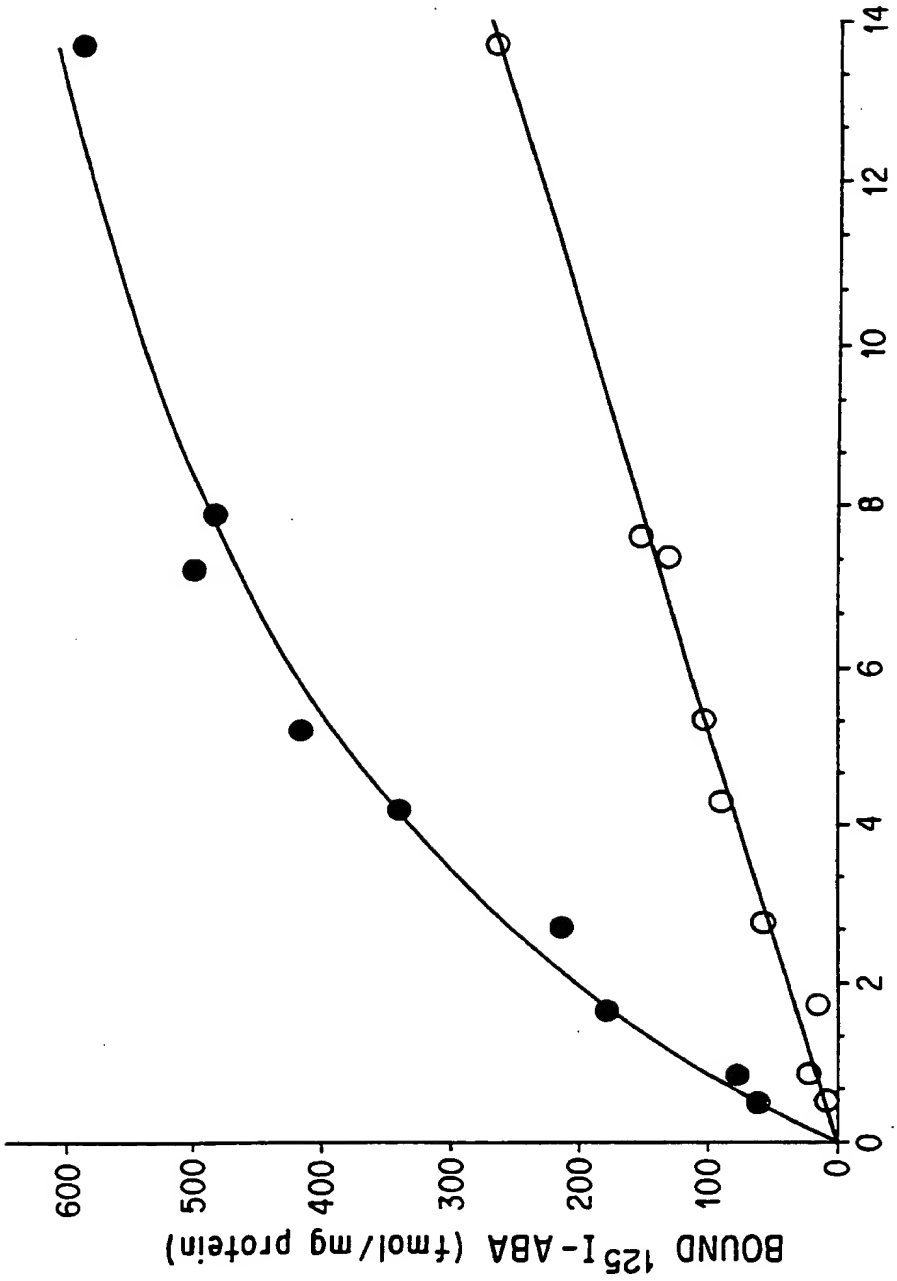

BOUND ^{125}I-ABA (fmol/mg protein)
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
FREE ^{125}I-ABA (nM)

FIG. 11A

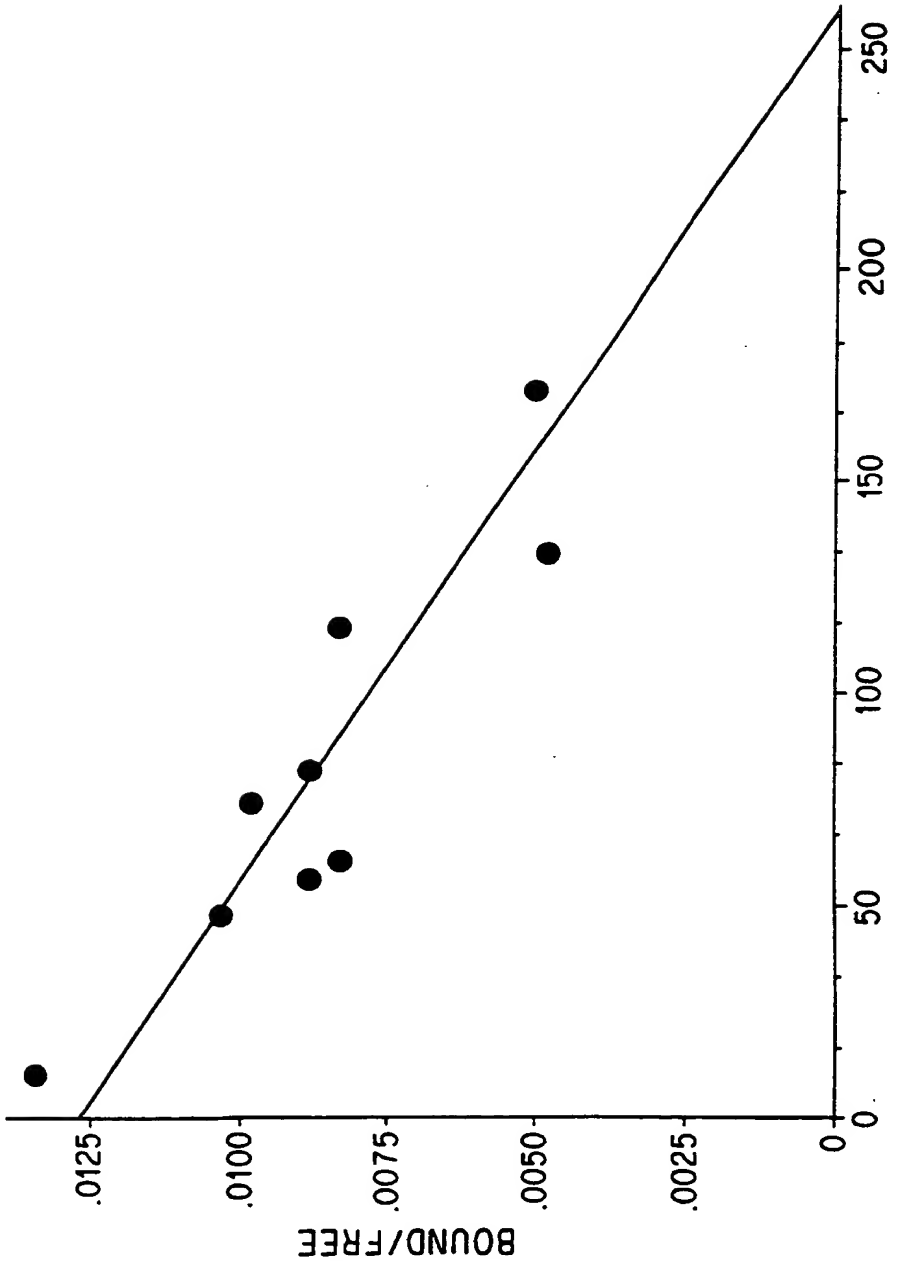

BOUND/FREE
.0125
.0100
.0075
.0050
.0025
0
0
50
100
150
200
250
BOUND (fmol/mg protein)

FIG. 11B

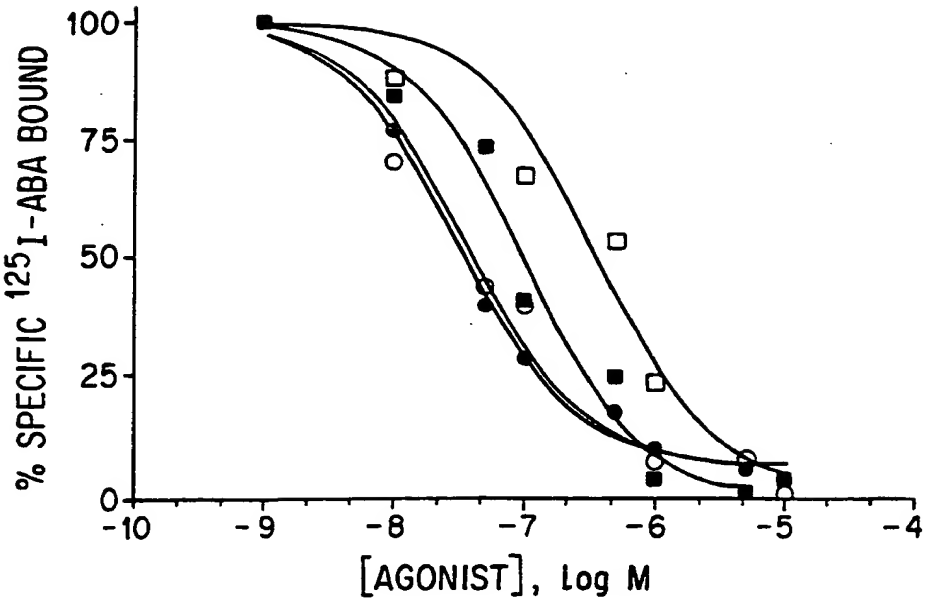


FIG. 12A

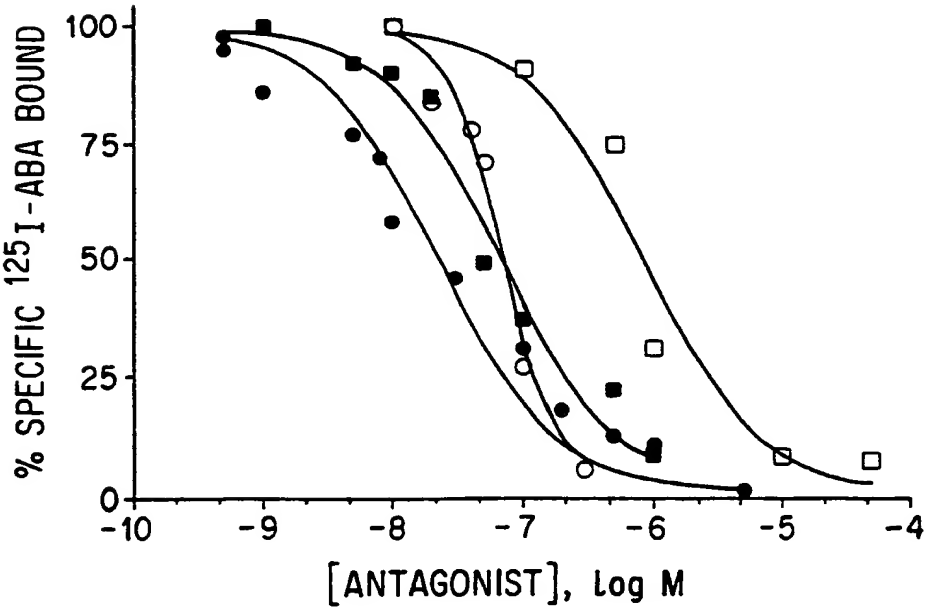


FIG. 12B

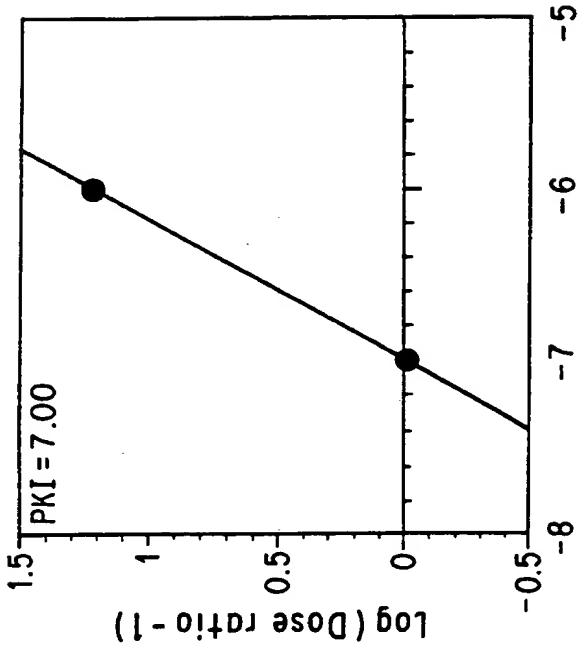


FIG. 13B

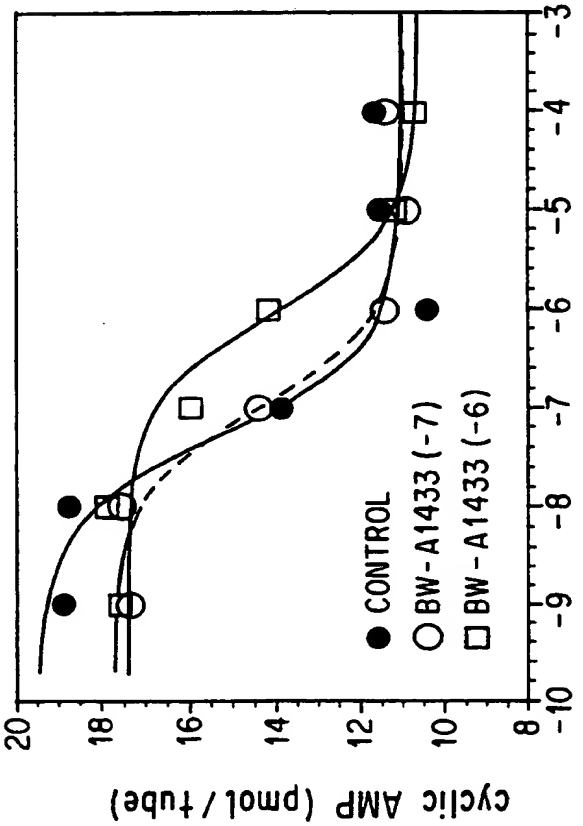


FIG. 13A

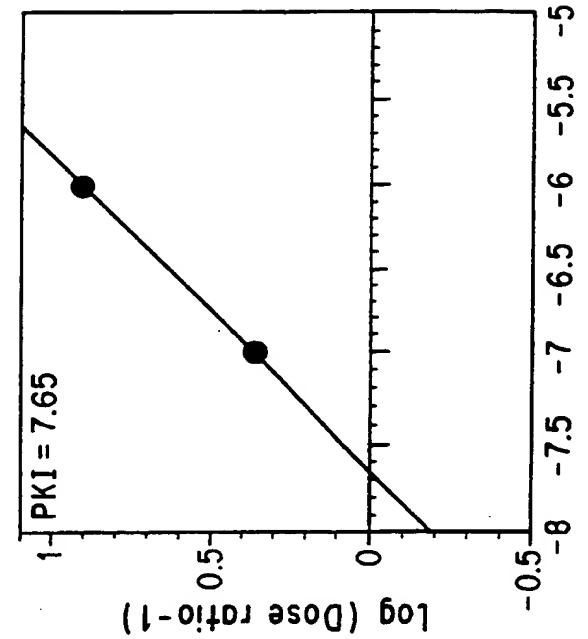


FIG. 13D

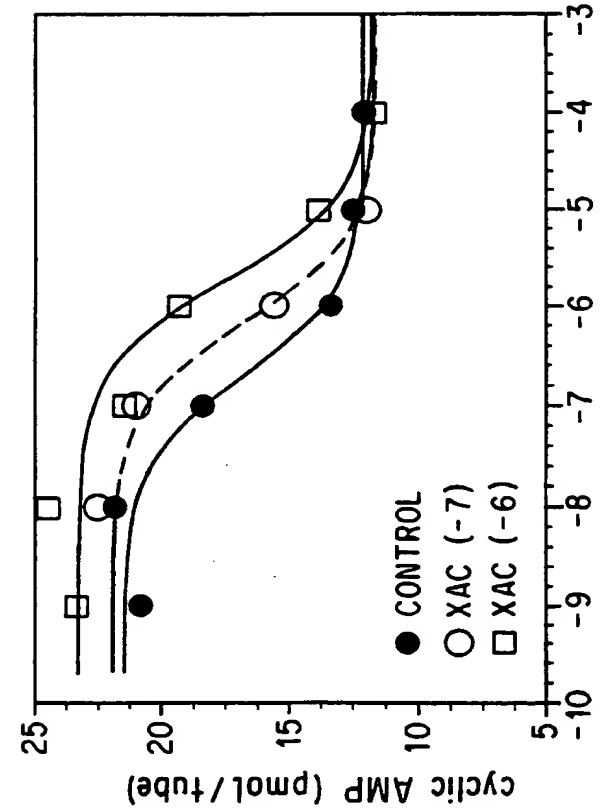


FIG. 13C

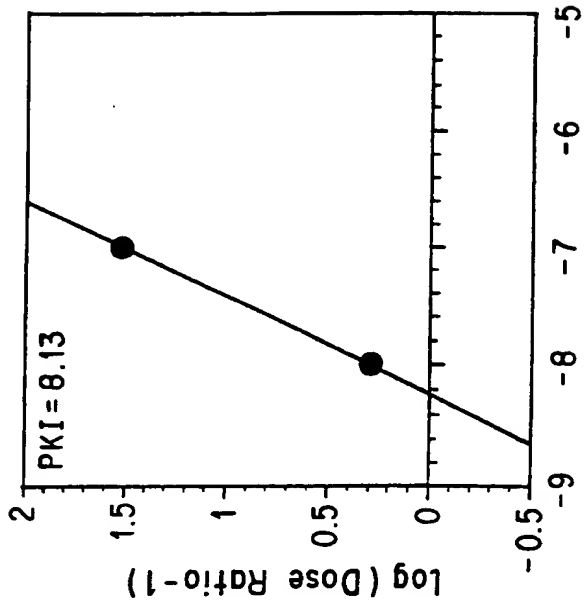


FIG. 13F

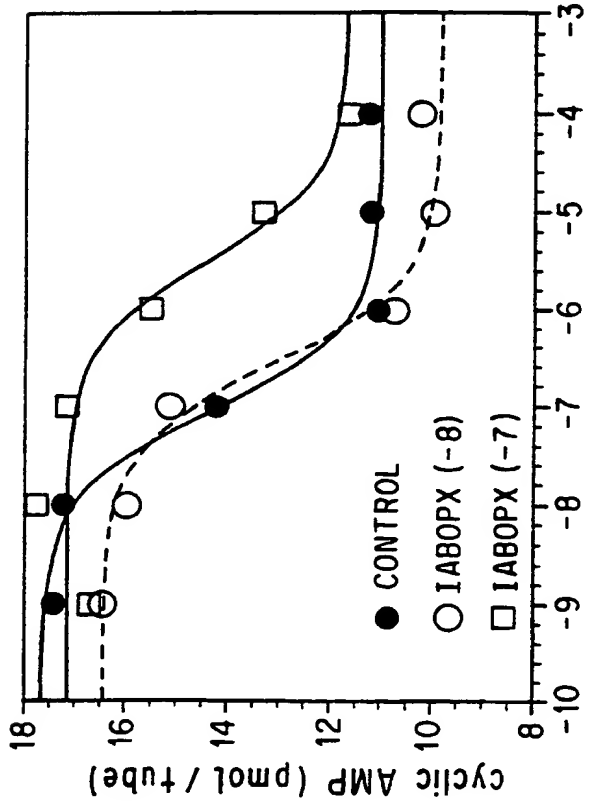


FIG. 13E

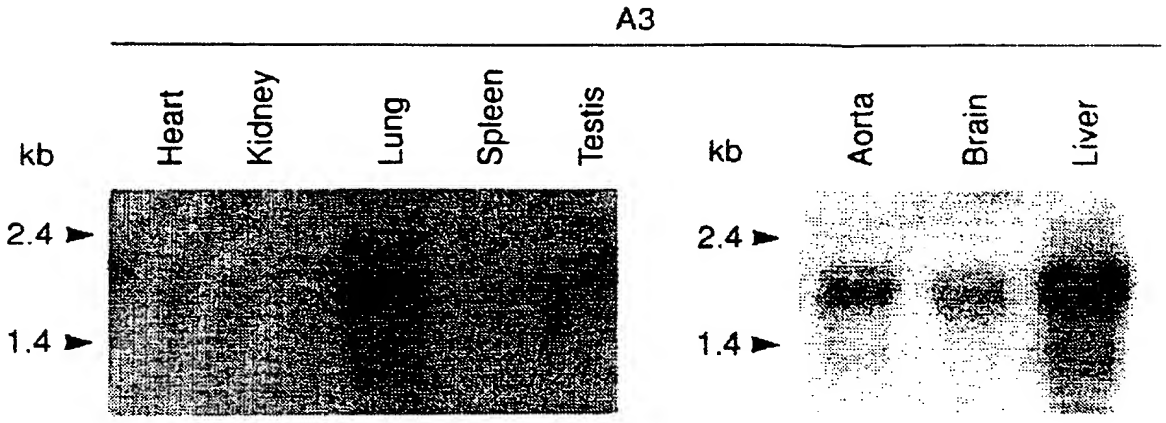


FIG. 14A

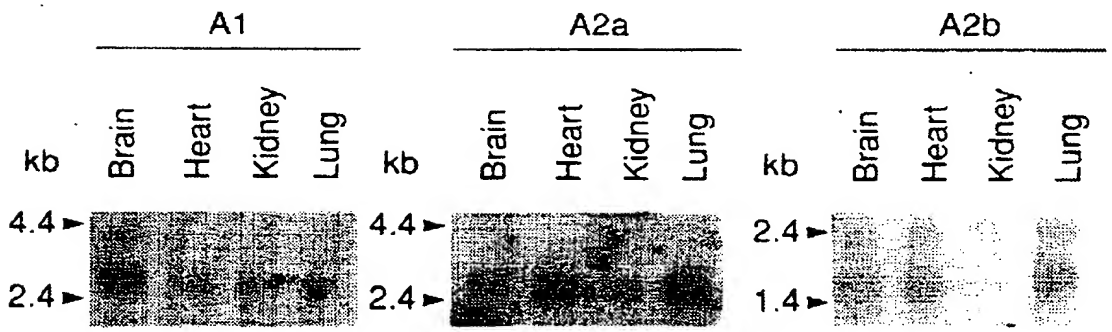


FIG. 14B

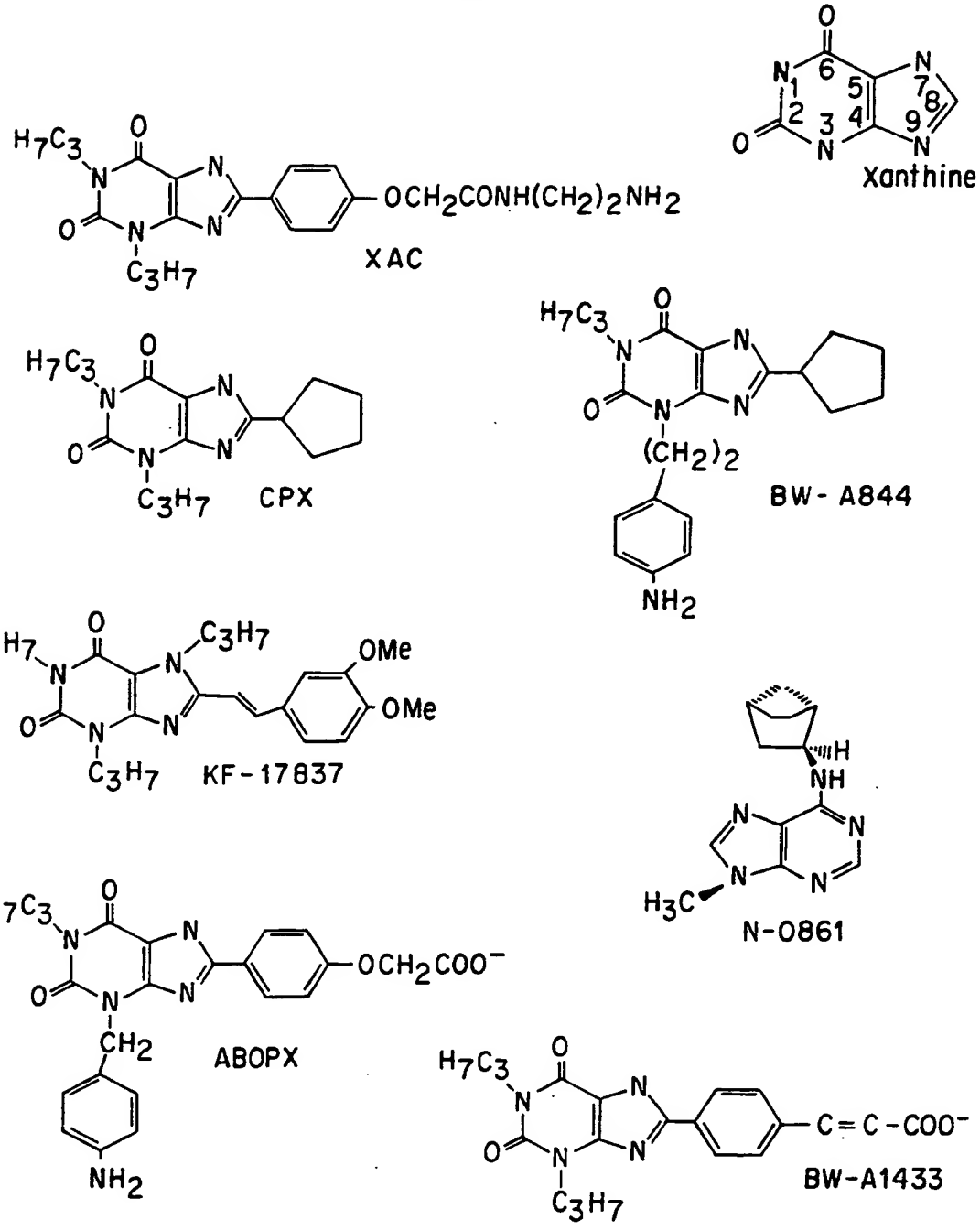


FIG. 15A

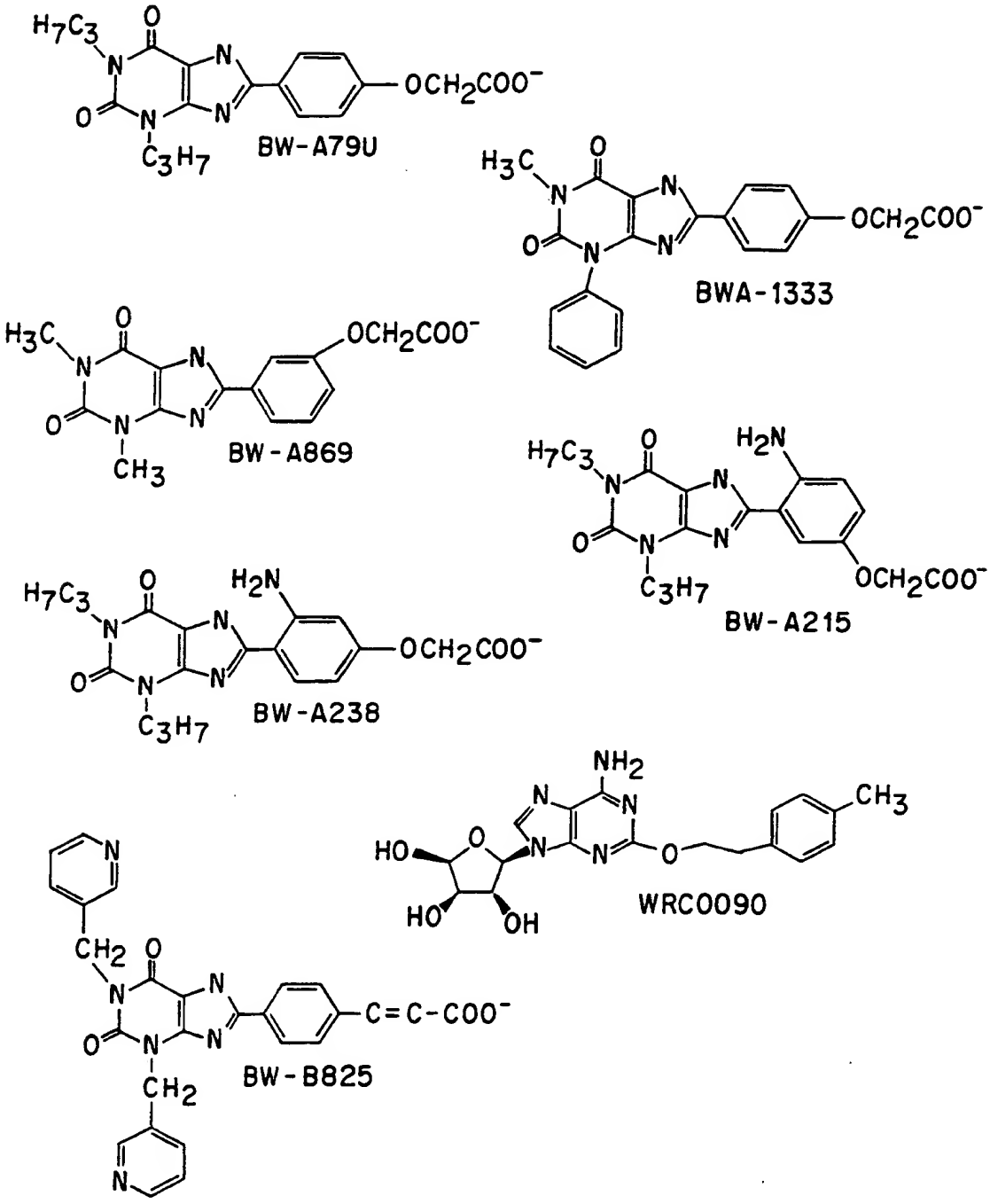


FIG. 15B

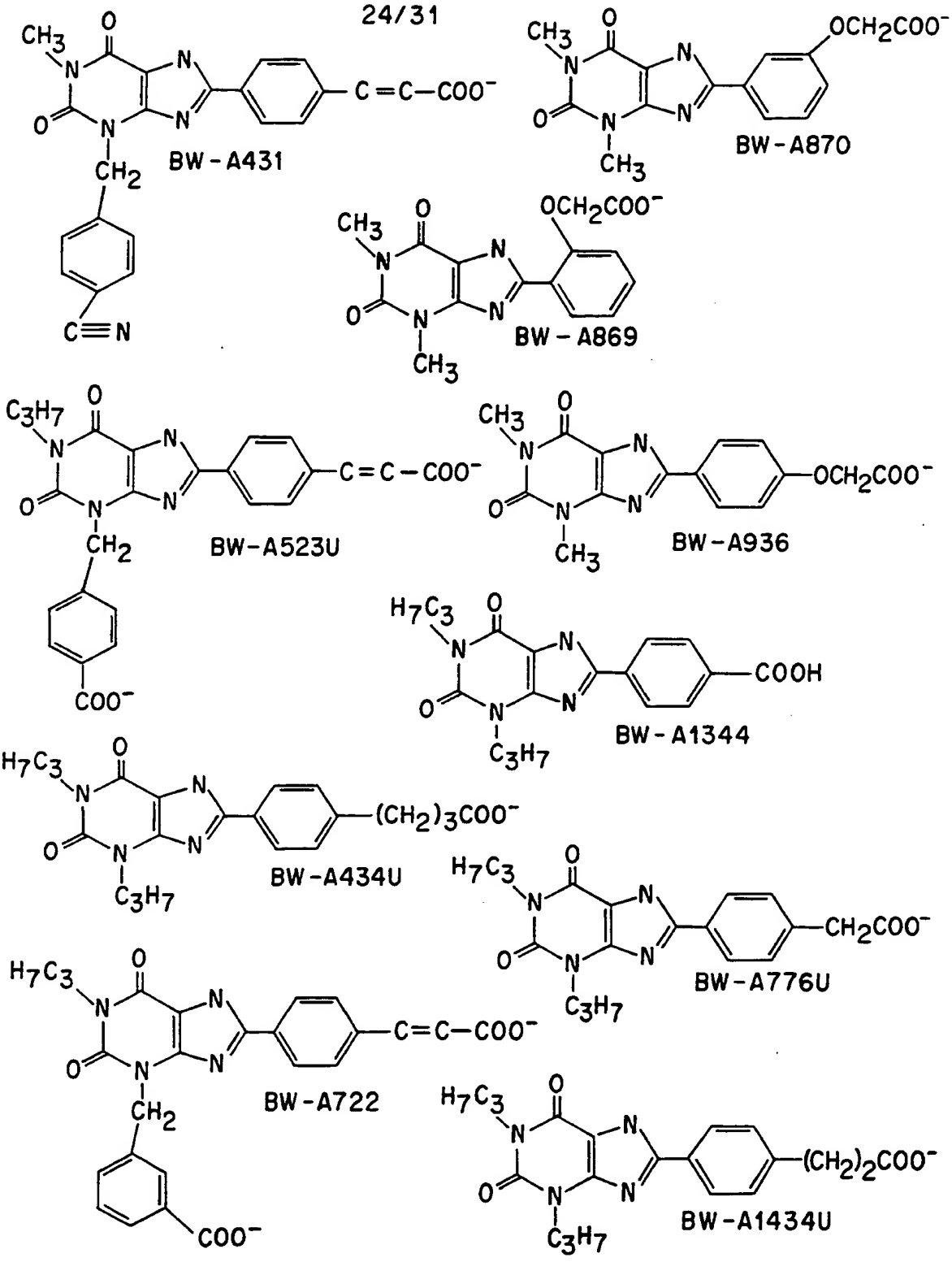

BW-A431
BW-A870
BW-A869
BW-A523U
BW-A936
BW-A1344
BW-A434U
BW-A776U
BW-A722
BW-A1434U

FIG. 15C

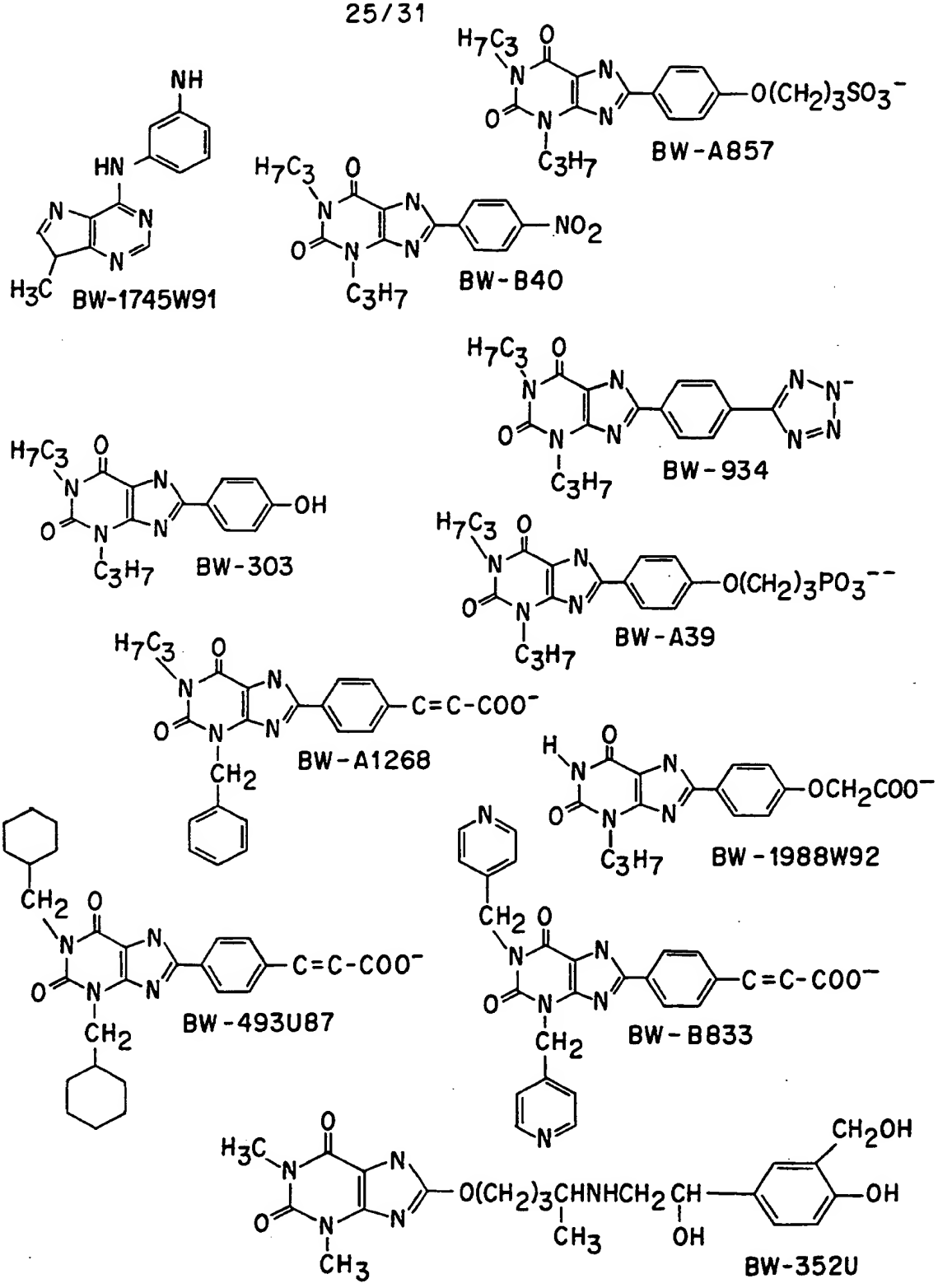

BW-A857
BW-1745W91
BW-B40
BW-934
BW-303
BW-A39
BW-A1268
BW-1988W92
BW-493U87
BW-B833
BW-352U

FIG. 15D

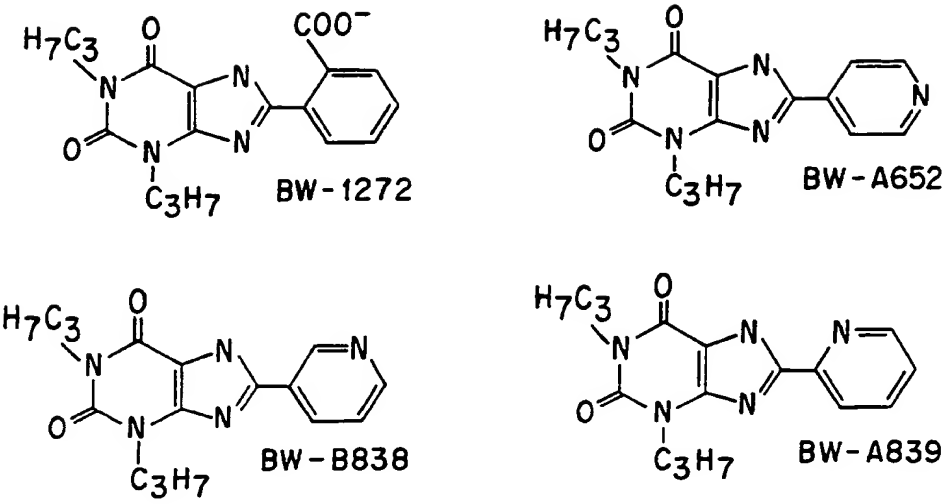


FIG. 15E

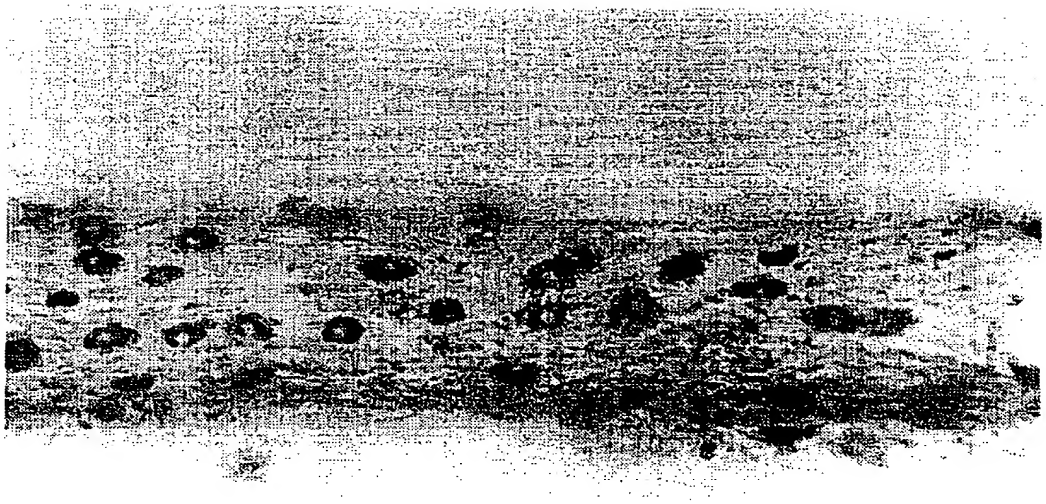


FIG. 16

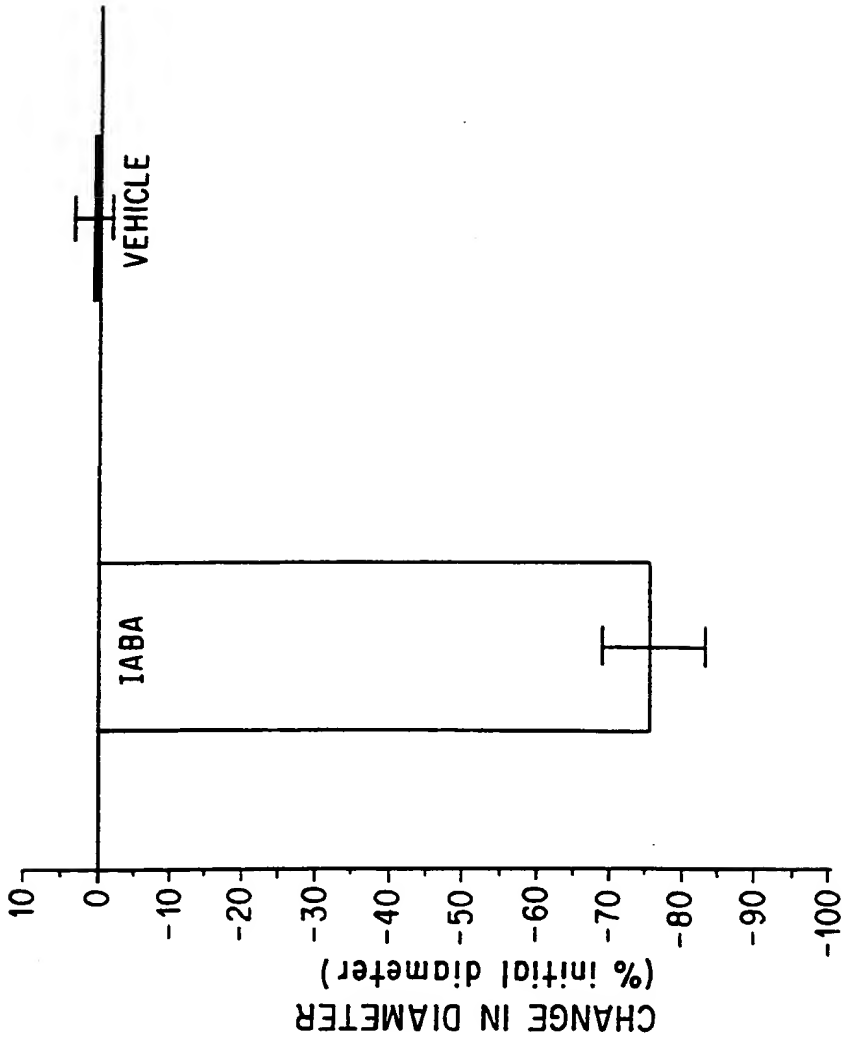

VEHICLE
IABA
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
CHANGE IN DIAMETER
(% initial diameter)

FIG. 17

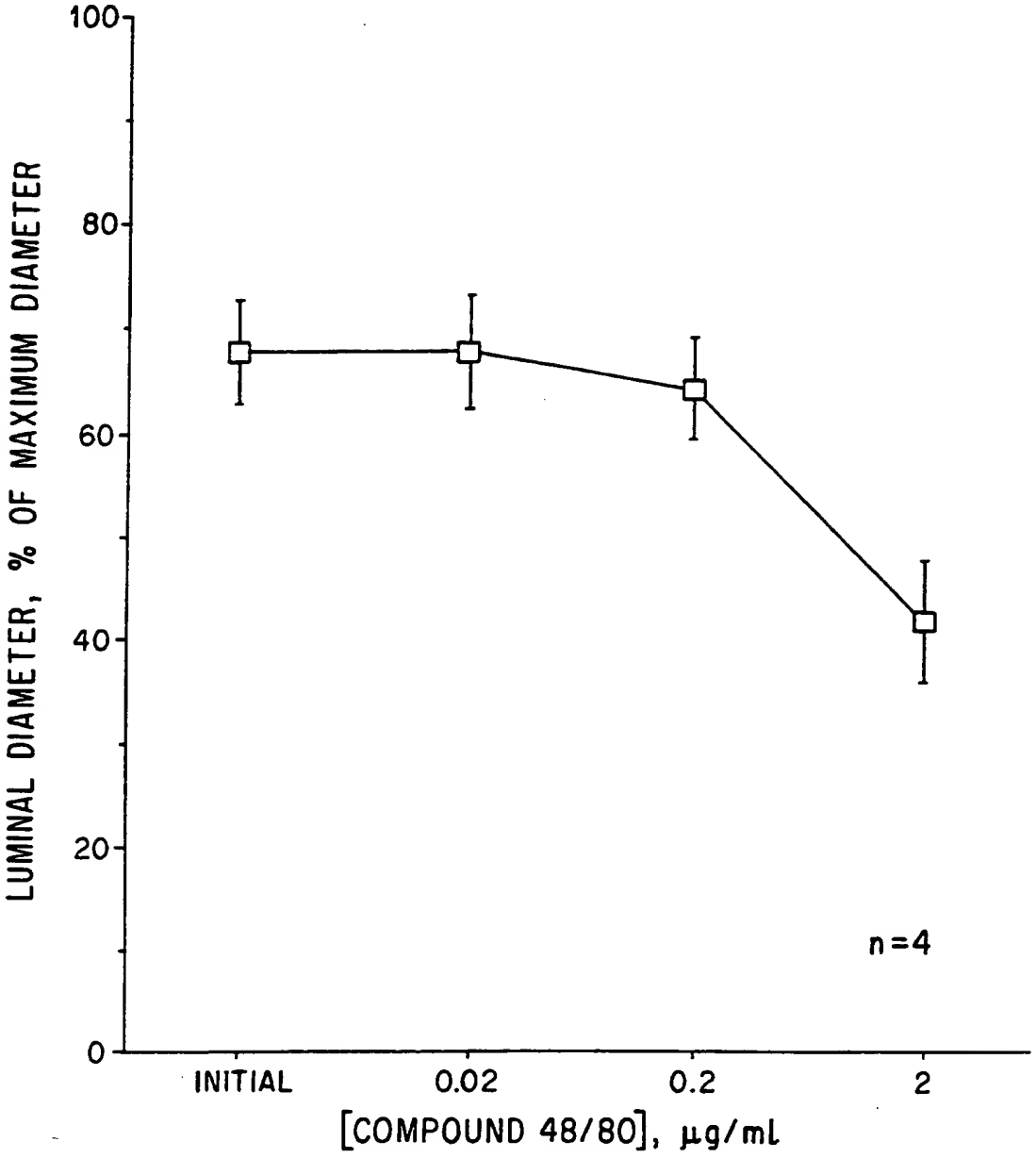

100
80
60
40
20
0
LUMINAL DIAMETER, % OF MAXIMUM DIAMETER
n=4
INITIAL
0.02
0.2
2
[COMPOUND 48/80], µg/ml

FIG. 18

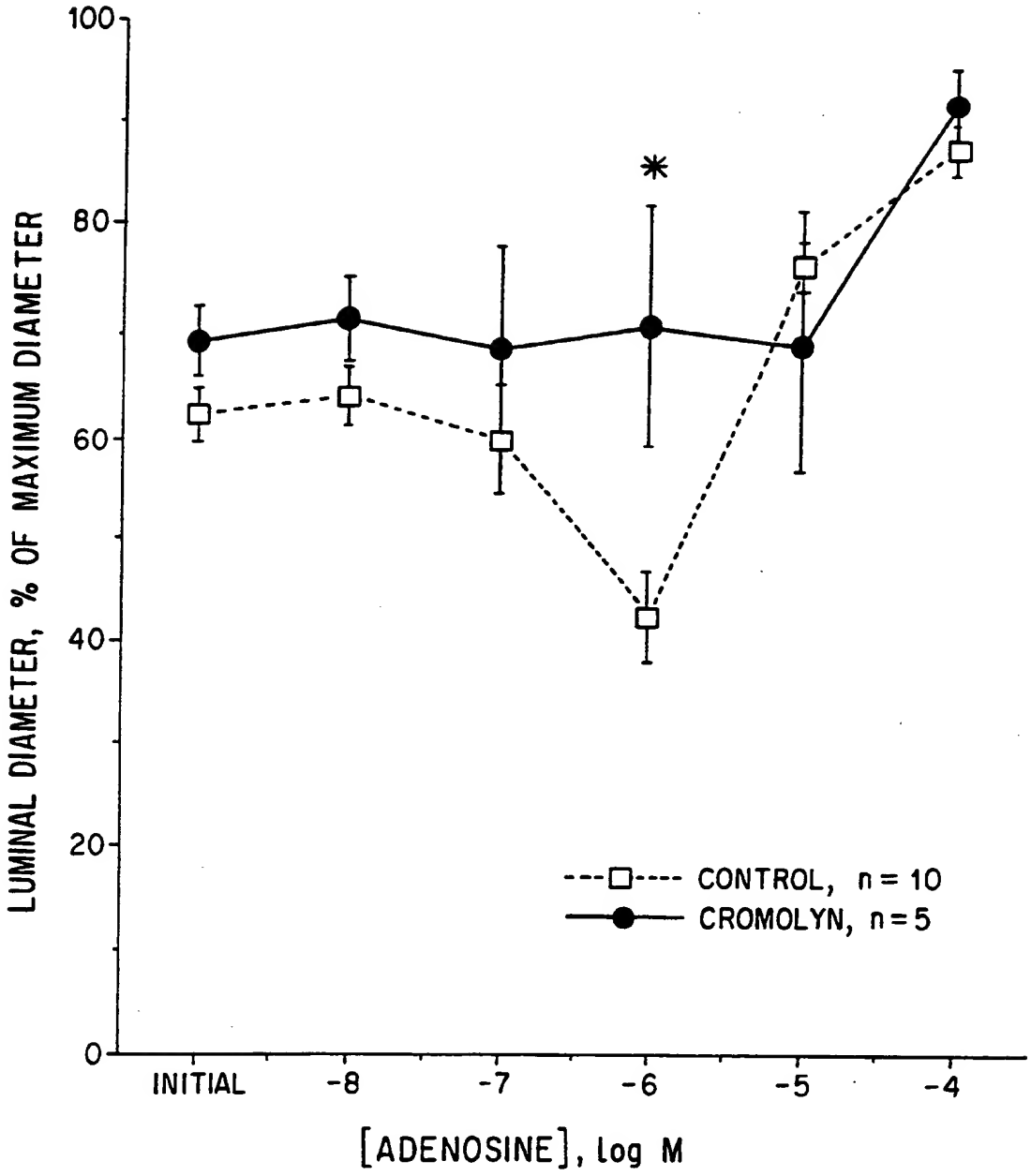

100
80
60
40
20
0
LUMINAL DIAMETER, % OF MAXIMUM DIAMETER
*
CONTROL, n = 10
CROMOLYN, n = 5
INITIAL
-8
-7
-6
-5
-4
[ADENOSINE], log M

FIG. 19

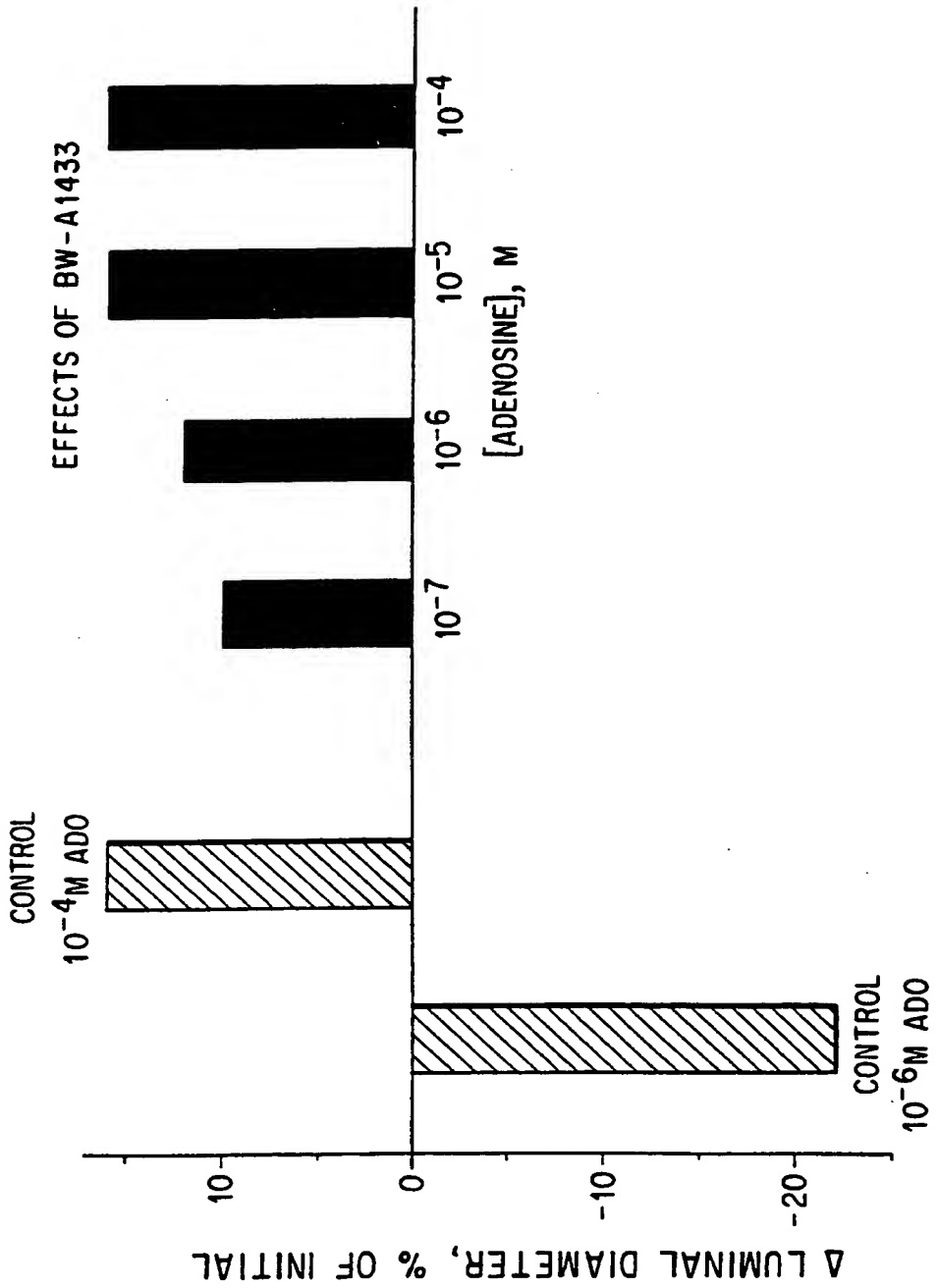

Δ LUMINAL DIAMETER, % OF INITIAL
10
0
-10
-20
CONTROL
10^{-4}M ADO
CONTROL
10^{-6}M ADO
EFFECTS OF BW-A1433
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[ADENOSINE], M

FIG. 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12272

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/52
US CL :514/263, 265, 826, 929
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/263, 265, 826, 929

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, BIOSIS, MEDLINE ONLINE
SEARCH TERMS: ADENOSINE RECEPTOR, A3,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,879,296 (DALUGE ET AL.) 07 NOVEMBER 1989, column 1, lines 30-41 and 57-66 and claim 19.	1,2,12,16-22
X,P	US, A, 5,298,508 (JACOBSON ET AL.) 29 MARCH 1994, see entire document, specifically column 3, lines 20-25.	1-3,12,16-22
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 80, issued April 1983, Bruns et al., "Adenosine receptor binding: Structure-activity analysis generates extremely potent xanthine antagonists", pages 2077-2080, see entire document, specifically abstract.	2-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 31 JANUARY 1995	Date of mailing of the international search report 09 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARY CEBULAK Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12272

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DRUG DEVELOPMENT RESEARCH, Volume 30, issued 1993, Carruthers et al., "Hypotensive responses to the putative adenosine A3 receptor agonist N6-2-(4-aminophenyl)-ethyladenosine in the rat", pages 147-152, see entire document.	1,16-22
X	BRITISH JOURNAL OF PHARMACOLOGY, Volume 109, No. 1, issued Mat 1993, Fozard et al., "Adenosine A3 recepetors mediate hypotension in the angiotensin II-supported circulation of the pithed rat", pages 3-5, see abstract.	16-22
X	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 268, No. 23, issued 15 AUGUST 1993, Ramkumar et al., "The A3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells", pages 16887-16890, see entire document.	3,16-22
X	JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, Volume 258, No. 3, issued 1991, Ali et al., "Methylxanthines block antigen-induced responses in RBL-2H3 cells independently of the adenosine receptors or cyclic AMP: evidence for the inhibition of antigen binding to IgE", pages 954-962, see entire document, specifically abstract.	16-22
Y	INTERNATIONAL ARCHIVE OF ALLERGY AND APPLIED IMMUNOLOGY, Volume 98, issued 1982, Ott et al., "Effects of adenosine on histamine release from human lung fragments", pages 50-56, see entire document.	1-3,12,16-22
X	MOLECULAR PHARMACOLOGY, Volume 44, issued 20 SEPTEMBER 1993, Linden et al., "Molecular cloning and functional expression of a sheep A3 adenosine receptor with widespread tissue distribution", pages 524-532, see entire document.	1,16-22
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 89, issued August 1992, Zhou et al., "Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor", pages 7432-7436, see entire document, specifically page 7436.	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12272

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1, 2, 3, 12, and 16-22

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12272

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. Claim 1, drawn to a method for achieving blockade of the vasoconstrictive response induced through adenosine activation of the A3 adenosine subtype.

II. Claim 2, drawn to a method for treating or preventing myocardial ischemia, inflammation, brain arteriole diameter constriction, and the release of allergic mediators.

III. Claims 3 and 12, drawn to a method for preventing or treating asthma, bronchoconstriction, allergic potentiation, inflammation or reperfusion injury in a human.

IV. Claims 4 and 13, drawn to a method for preventing mast cell degranulation in a human.

V. Claims 5-7, and 14-15, drawn to a method for ahcieving blockade of vascular constriction induced through activation of the A3 subtype of the adenosine receptor in a primate.

VI. Claims 16-22, drawn to a method for achieving blockade of the A3 subtype of the adenosine receptor in a primateby contacting the recpetor with a xanthine or xanthine derivative.

VII. Claim 23, drawn to a method for treating an autoimmune disease.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or correspondingspecialtechnicalfeatures for the following reasons: the methods are related in so much as they utilize the same materials but each is drawn to a different therapeutic method, i.e., there is more than one inventive concept.